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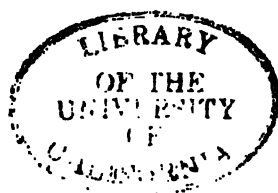
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CRYSTALLINE URIDINPHOSPHORIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 28, 1919.)

In previous communications¹ crystalline salts of uridinphosphoric acid were described. They were the brucine salt, two ammonium salts, the lead, and the barium salt. Nearly every one has its value in the process of preparation of the free uridinphosphoric acid. The insolubility of the brucine salt is the property on which is based the separation of this from a mixture containing other nucleotides. The ammonium salt is the transition step from the brucine salt to every other salt; the lead salt serves for separation of the nucleotide in its pure state, and hence for its identification when the nucleotide is available in only small quantities. For final identification the crystalline nucleotide offers the most satisfactory material. It was prepared in the following manner.

The diammonium salt described in the previous communication was converted into the lead salt. This was suspended in water, and through the suspension a stream of hydrogen sulfide gas was passed. The filtrate from lead sulfide was freed from hydrogen sulfide by distillation under diminished pressure at room temperature. To the clear solution of the nucleotide a solution of neutral lead acetate was added to form again the lead salt. The lead salt was again treated with hydrogen sulfide as before, the resulting clear solution was concentrated under diminished pressure to a small volume, and then placed in a vacuum desiccator, where it was allowed to concentrate slowly under diminished pressure. When the solution attained the thickness of glycerol it was dissolved in hot 99.5 per cent alcohol and again placed in a vacuum desiccator and allowed to concentrate under diminished

¹ Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21; *J. Biol. Chem.*, 1918, xxxiii, 229; 1919, xl, 395.

pressure over sulfuric acid. This operation was repeated many times and finally the thick syrup crystallized into a nearly solid, sticky mass. In order to separate the crystals from the viscous mother liquor the material was triturated with a very small quantity of hot anhydrous methyl alcohol. The crystalline material was then washed with cold methyl alcohol, and finally the substance was suspended in dry methyl alcohol. The mixture was brought to a boil and then the crystals were filtered off. The mother liquors and the wash alcohol on standing under diminished pressure over sulfuric acid gave a second crop of crystals. The substance had the melting point M. P. = 202° (corrected) with decomposition and the following composition.

0.1000 gm. of the substance gave 0.1231 gm. of CO_2 and 0.0376 gm. of H_2O .
 0.2000 " " " " employed for Kjeldahl nitrogen estimation required 12.36 cc. of 0.1 N acid for neutralization.
 0.3000 gm. of the substance gave 0.1020 gm. of $\text{Mg}_2\text{P}_2\text{O}_7$.

	Calculated for $\text{C}_6\text{H}_{11}\text{N}_3\text{P}_2\text{O}_8$ per cent	Found. per cent
C	33.32	33.40
H	4.05	4.18
N	8.64	8.65
P	9.58	9.48

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.21 \times 100}{1 \times 2} = +10.5$$

Thus every one of the four nucleotides composing the molecule of yeast nucleic acid has now been prepared in crystalline form.

THE DETERMINATION OF SACCHARIN IN URINE.

By GEORGE S. JAMIESON.

(From the Bureau of Chemistry, U. S. Department of Agriculture.)

(Received for publication, November 25, 1919.)

The method to be described is based upon the quantitative extraction of the saccharin with ether from the acidified urine which had been previously treated with normal lead acetate and filtered. The sulfur of the extracted saccharin was determined as barium sulfate, after the fusion of the residue from the ether with sodium carbonate. The amount of saccharin present was found by multiplying the weight of the barium sulfate by 0.7844. Ether was chosen as the most suitable solvent because it had been shown by Marden¹ that saccharin could be readily extracted in a quantitative manner with ether from aqueous solutions which had been acidified with hydrochloric acid. For references upon the determination of saccharin the reader may consult the researches described by Gnadinger,² and Seeker and Wolf.³

It has been found by employing the modification of the sulfur method used for the determination of saccharin in food substances, described below, that satisfactory results can be obtained for amounts of saccharin varying from 1 mg. upwards per 100 cc. of urine. Smaller amounts of saccharin could probably be estimated with some accuracy by using larger quantities of urine for the analysis. A detailed description of the procedure will be given so that other analysts may obtain accurate results with this method.

In order to test the method, a standard solution was prepared which contained 1 mg. of saccharin per cc. The saccharin used was a high grade commercial product which had been previously analyzed in order to determine its purity. It melted at about

¹ Marden, J. W., *J. Ind. and Eng. Chem.*, 1914, vi, 315.

² Gnadinger, C. B., *J. Assn. Off. Agric. Chem.*, 1917, iii, 23-33.

³ Seeker, A. F., and Wolf, M. G., *J. Assn. Off. Agric. Chem.*, 1917, iii, 38-43.

220°C. The ignition of about 0.2 gm. on a platinum foil left no perceptible residue. The amount of actual saccharin present in the sample was determined by the well known Reid method⁴ and it was found to contain 98.79 per cent (average of two closely agreeing analyses). On the basis of the analyses, 2 liters of solution were prepared so that 1 cc. should contain 1 mg. of saccharin. This solution was standardized by determining the amount of saccharin present in measured volumes by the sulfur method described below. The average of two closely agreeing analyses gave the value 1 cc. = 0.00100 gm. of saccharin, which is in agreement with the results previously obtained by the Reid method. Measured quantities of the standard saccharin solution were added to 100 cc. portions of urine which was sometimes fresh and at other times 1 or 2 days old. To each solution, 10 cc. of a 20 per cent normal lead acetate solution were added and after thorough stirring the solution was allowed to stand for an hour before filtration. It was found essential to heat urine which contained albumin almost to boiling for a few minutes, and then add the lead acetate solution, otherwise unbreakable emulsions were formed when the aqueous solution was agitated with ether to extract the saccharin. After heating, the solutions were allowed to stand for 1 hour before filtration as previously described.

Since it is important to add a slight but distinct excess of lead acetate over that required for the precipitation of the compounds which if left in the solution would interfere with the extraction of the saccharin by ether, by causing the formation of emulsions, it is necessary to make some preliminary experiments to determine the proper volume of lead acetate required for each urine. It has been observed that a weak urine requires somewhat less lead acetate than a strong, highly colored urine. However, after a little experience, one can closely estimate the proper amount of lead acetate required, in the majority of cases, from the color of the urine to be examined. On the other hand, if more than a slight excess of lead acetate over the required amount was present, the precipitated lead chloride formed when the solution was strongly acidified with hydrochloric acid previous to the ether extractions was found to interfere with the complete recovery of

⁴ Reid, E. E. *Am. Chem. J.*, 1899, xxi, 461.

the saccharin present. The solution was filtered by suction through a mat of paper pulp about 0.5 cm. thick. The paper pulp was prepared by stirring filter clippings with enough concentrated hydrochloric acid to moisten it until it was thoroughly disintegrated. Then about 15 volumes of water were added and, after shaking, the mixture was ready for use. When the proper thickness of mat had been obtained in a 6 cm. funnel, it was washed three times with about 50 cc. of water each time to remove the acid. When the lead precipitate was in the funnel and most of the solution had filtered through, it was washed four times, using about 10 cc. of water each time. During the first part of the filtration, a gentle suction was used, but when the precipitate was ready for washing, the full suction was used. The suction was continued until no more filtrate was obtained even after the precipitate had been firmly pressed to a hard mass with a spatula. It should be added that the funnel and precipitate were washed again with a small quantity of water after the precipitate had cracked just before pressing it down with the spatula. It was found that the filtration, including the washing and pressing of the precipitate, usually required about 1 hour. It is most important that every piece of apparatus used in the analysis be rinsed absolutely free from sulfates and furthermore the analysis must be made in a room where sulfuric acid is not volatilized. The filtrate was transferred to a suitably sized separatory funnel and the filtering flask was rinsed twice with about 5 cc. of water. Then 15 cc. of concentrated hydrochloric acid were added and, after mixing, 55 cc. of ether were added. The contents of the funnel were shaken for 2 minutes. After the layers had separated for about 10 minutes, the aqueous solution was drawn into a clean beaker. The ether was rotated in order to wash the sides of the funnel as free as possible from water. When the water had settled to the bottom of the funnel, it was tapped off. The ether was poured into a 600 cc. beaker and evaporated by directing a gentle current of air on the surface. The aqueous solution was returned to the funnel and extracted again with 50 cc. of ether in precisely the same manner as before. The second ether extract was added to the beaker containing the first extract and a third extraction using the same volume of ether was made. When the combined ether extracts had been evaporated as completely as

possible with the air current, the remaining moisture was removed by heating the beaker on a steam bath. Care was taken not to heat so strongly as to cause the solution to boil. It is very important not to allow a single drop of aqueous solution to be carried along when transferring the ether to the beaker from the separatory funnel. The residue from the ether was allowed to cool to room temperature and treated with about 12 cc. of absolute ether by pouring it around the upper edge of the beaker so as to wash down the saccharin adhering to the sides. The ether was allowed to remain on the residue for 5 minutes; then it was transferred to a 30 cc. nickel crucible. While the ether was being evaporated by a gentle current of air directed on the surface of the liquid, the residue was extracted again with 12 cc. of absolute ether. This ether extract was added to the crucible. A third portion of about 15 cc. of absolute ether was added to the residue which was detached from the sides and bottom of the beaker by rubbing with a stout glass rod. A rubber-tipped rod must not be employed. This extract was filtered to remove the suspended sediment through a 5 cm. filter into the crucible. The beaker was rinsed and the residue was stirred with another 15 cc. portion of ether which was filtered and added to the crucible. Then the filter was washed by adding about 5 cc. more of ether around the upper edge. When the ether had evaporated, about 2 gm. of anhydrous sodium carbonate were added. The sodium carbonate was spread, by means of a small spatula, up the sides of the crucible as far as the residue extended. The nickel crucible was placed in a porcelain crucible and heated for 10 minutes with a low flame. Then the flame was gradually increased to its full capacity. At this point, the porcelain crucible was removed and the nickel crucible was heated directly for 5 minutes at the highest temperature of the burner. When the crucible was cold, 20 cc. of water were added and, after standing for about 10 minutes, the contents of the crucible were heated almost to boiling. The solution was filtered through a 9 cm. washed filter and the crucible was rinsed several times with warm water. After washing the filter four times with water, the beaker containing the filtrate was covered and the solution was acidified with 5 cc. of hydrochloric acid. The solution was heated for 5 minutes on the steam bath before adding 10 cc. of a 10 per cent solution of barium chloride to

precipitate the sulfate. When the solution had been heated for about 3 hours, it was allowed to cool to about 50°C. It was filtered on a carefully prepared Gooch crucible and washed with 60 to 75 cc. of water. In filtering small amounts of barium sulfate, it was found best to use a very gentle suction until after the final washing had ceased running through. At this point the suction was increased very gradually until the maximum was reached. The crucible containing the precipitate was placed in a porcelain crucible and heated for a short time with a small flame. Then it was heated with the full flame for 5 minutes. When the crucible

TABLE I.

No.	Saccharin taken with 100 cc. urine.	Barium sulfate found.	Saccharin cal- culated.	Error.
	gm.	gm.	gm.	gm.
1	0.0120	0.0149	0.0117	-0.0003
2	0.0120	0.0153	0.0120	0.0000
3	0.0080	0.0099	0.0078	-0.0002
4	0.0080	0.0102	0.0080	0.0000
5	0.0005	0.0007	0.0005	0.0000
6	0.0051	0.0064	0.0052	+0.0001
7	0.0040	0.0050	0.0039	-0.0001
8	0.0030	0.0040	0.0031	+0.0001
9	0.0010	0.0015	0.0012	+0.0002
10	0.0020	0.0027	0.0021	+0.0001
11	0.0020	0.0032	0.0024	+0.0004
12	0.0030	0.0040	0.0031	+0.0001
13	0.0050	0.0060	0.0047	-0.0003
14	0.0050	0.0062	0.0050	0.0000
15	0.0100	0.0126	0.0099	-0.0001

had cooled for 20 minutes it was weighed. The weight of barium sulfate obtained after deducting the blank correction described below was multiplied by 0.7844 to obtain the weight of saccharin which it represented. A blank analysis for sulfate was made using all the reagents in the same amounts as in the actual experiments and less than 0.1 mg. of barium sulfate was obtained. Several blank analyses were made at various times carrying out the entire process with 100 cc. portions of urine as described above. The average of the blank analyses amounted to 0.7 mg. of barium sulfate which has been deducted from the weights of barium sulfate given in Table I.

From the table of analyses it will be observed that very satisfactory results were obtained. In order to obtain good results, it is most essential that the directions given above should be executed with the greatest care possible. Furthermore, before attempting to apply this method a sufficient number of blank analyses should be made, if possible, upon urine of the subject to be experimented upon, so that the proper correction can be applied in connection with the determination of saccharin.

SUMMARY.

Urine is treated with a solution of lead acetate and filtered. After acidifying with hydrochloric acid, the saccharin is extracted by ether, the ether removed by evaporation, and the saccharin extracted from the residue with ether. The ether is removed and the resulting residue is fused with sodium carbonate and the sulfur determined as barium sulfate. The amount of saccharin is calculated from the sulfur found after making a correction for the blank. The experimental results obtained by the analysis of urines, to which known amounts of saccharin had been added, show that the method is capable of giving satisfactory results providing that the directions are followed exactly as described in every detail.

A THERMOREGULATOR WITH THE CHARACTERISTICS OF THE BECKMANN THERMOMETER.

By R. B. HARVEY.

*(From the Bureau of Plant Industry, United States Department of
Agriculture, Washington.)*

PLATE 1.

(Received for publication, November 17, 1919.)

In working at low temperatures in a constant temperature chamber it was found desirable to reduce the heat capacity of the thermoregulator as much as possible in order to give quick response to small changes in temperature of the air. It was found advantageous also to have the thermoregulator show the same lag effects as the standard measuring instrument, the Beckmann thermometer.

The instrument here shown (Plate 1) can be set easily at any temperature desired between -20 and $+250^{\circ}\text{C}$. Approximate settings can be made by warming or cooling the mercury bulb until the upper level of the column reaches the desired point on the setting scale. For making more accurate settings a slight blow will separate a series of small globules of mercury from either portion after the mercury is divided approximately by means of the scale. As many of these droplets as are desired can be added to the contents of the bulb by turning the instrument upside down. The capillary is sufficiently large to allow the mercury in the bulb to flow through it easily when the column is broken by a slight blow with a pencil. The instrument is thus more easily set than the ordinary Beckmann thermometer.

The lower platinum contact should be made in an enlargement of the capillary to prevent sticking of the mercury at that point. The upper contact is placed at the end of the capillary so that small temperature changes will make or break the circuit. The mercury column is of sufficient diameter to carry the current required to operate a 150 ohm relay using 2 or 4 volts. The heating circuit should be broken by a relay.

When run on test in the air bath with a standard Beckmann thermometer the air temperature was regulated within $\pm 0.004^{\circ}\text{C}$. when stirred vigorously. The accuracy of regulation of the air temperature varies greatly with the heat capacity of the source of heat and the rate of stirring.



(Harvey: Thermoregulator.)



THE DETERMINATION OF HYDROGEN ION CONCENTRATION.

By JOHN W. M. BUNKER.

(From the Department of Bacteriology, Digestive Ferments Company, Detroit.)

(Received for publication, November 12, 1919.)

The reasons for the measurement of reaction of culture media and biologic fluids in terms of pH are well covered by numerous publications upon the subject. The art of making the determination is still susceptible of great variations in method. Certain principles concerning the technique of electrolytic determinations are important. The electrode itself should be as simple in construction as possible, in order that it may be easily cleaned and easily replaced. The measurement of small quantities of test liquid should be possible. Easy attainment of equilibrium in the solution should be expected. Those parts of the apparatus which come in contact with test liquids should be easily subjected to sterilization.

An electrode and vessel are here described which fulfil some of these requirements. In its design, ideas have been borrowed freely from published work and through personal observations. It is a modification of the bubbling electrode, used in a closed vessel.

The tube used to conduct the hydrogen gas to the platinum wire of the hydrogen electrode is the essential part of this apparatus. It is indicated by E in Fig. 1. This is a piece of glass tubing of $\frac{5}{16}$ inch internal bore with a side arm of small diameter joined to it at approximately 1 inch from the top. The $\frac{5}{16}$ inch tubing is about 5 inches long over all. In its preparation it is sealed at the bottom and then heated on one lower "corner," and a bubble at this point is blown and later broken with a file. The edges of the lip which remain are fire-polished, and then the end of the tube is heated and the round hole thus obtained is contracted to an approximately oval shape.

The hydrogen electrode itself, B, is made from a piece of $\frac{1}{8}$ inch tubing with a short piece of platinum wire sealed in at the bottom, projecting outside about $\frac{1}{8}$ inch, and inside far enough to make contact with mercury C, with which the lower part of this tube is filled. A small rubber stopper A is fitted tightly around this tube at about 1 inch from the top. The entire electrode is about 6 inches long. In assembling the apparatus, the electrode tube B

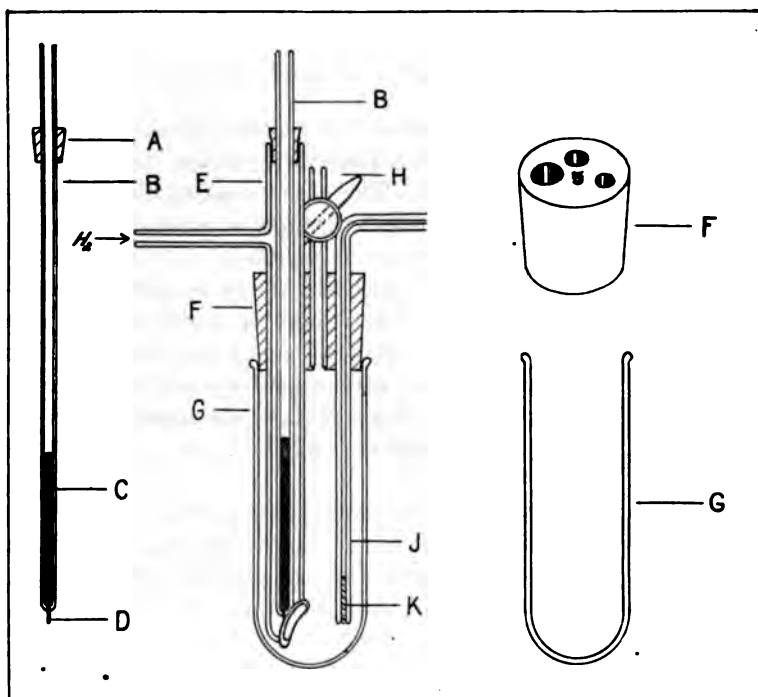


FIG. 1. Diagram of hydrogen electrode.

with its stopper A is inserted in tube E and pushed down tightly, and so adjusted that the platinum wire D is opposite the orifice in the lower end of the tube E. The exact point of this must be determined by trial, as must the size and shape of the orifice. When the assembled electrode is dipped in a liquid, and gas is bubbled through it in the direction indicated, there should form a large bubble at the base of the tube E which is held together

by the walls of the tube until it has travelled far enough for the level of the liquid within the tube to have reached a point below the platinum wire, thus exposing the wire to hydrogen gas. The bubble is pushed out of the tube and, as it leaves, the liquid rushes in behind it up into the tube, and should again bathe the platinum needle in liquid. This gives the effect of the rocking electrode of Clark,¹ in which the platinum electrode itself is bathed alternately in liquid and in gas. If the orifice is too wide, the bubble will be pushed out before it has travelled far enough to clear the platinum needle, and in that case the tube E must be dried, reheated in the flame, and the opening constricted.

The electrode vessel G which we call a pH tube, is an ordinary test-tube of 1 inch internal diameter, and approximately 3 inches in length. The exact dimensions are not important. We have a supply of these tubes on hand, carefully washed and dried properly, and stored away ready for determinations. When a series of determinations of pH is to be made, the proper number of tubes is selected, each is given its proper liquid, and is set up in a rack ready to use. One by one each may then be pushed up under the hydrogen electrode against the rubber stopper F and, as soon as the determination is read, slipped out to make way for the next.

The rubber stopper F is a No. 5 stopper with three holes made by cork borers to receive tightly the tube E and the outlet tube H with its stop-cock, and to receive not so tightly, the potassium chloride arm J, which is made of small capillary tubing. The rubber stopper is slipped up over the hydrogen electrode tube E and fastened to it with cement or shellac. The outlet tube H which carries a stop-cock is inserted into the rubber stopper, and also cemented in place. The stopper remains on the tube E at all times. The capillary tube J plugged at the end with a bit of cotton wicking K makes the connection between the saturated potassium chloride of the calomel half-cell and the test liquid. The tube J slips up and down in the stopper and should fit as tightly as possible, still allowing the necessary motion. The apparatus is assembled as in Fig. 1. Hydrogen gas is bubbled through the inlet tube for approximately 30 seconds at a rapid

¹Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.

rate, with the outlet tube H open and the potassium chloride arm J lifted out of the test liquid. Then the stream of hydrogen is slowed down to such a rate that the bubble travels in the lower end of the tube E once every 2 seconds. This is maintained for about 1 minute. The relation of the parts, the volume of liquid, and the size of the bubble of hydrogen liberated are such that adequate stirring of the test liquid is obtained by the bubbling, and it is not necessary to shake or rock the apparatus. At the end of the bubbling the hydrogen is shut off, by an external stop-cock, just after a bubble of gas has been liberated from the tube E and the liquid has assumed a level in the tube above the platinum needle D. The stop-cock in the outlet tube is immediately closed, thus insuring an atmosphere of hydrogen gas over the test liquid in a closed vessel. The potassium chloride arm J is then lowered so that contact is made, and reading is taken by whatever electro-metric apparatus is available.

If it is desirable to avoid all chance of contact-potential error, the wicking K in the potassium chloride arm J may be dispensed with, and, after the arm J is lowered to the bottom of the pH tube E, potassium chloride may be run in under the test solution in order to make a wide zone of contact between the potassium chloride and the test solution, the arm J, of course, being lowered to make contact with the volume of potassium chloride in the bottom of the tube G, and the stop-cock outside of the apparatus closed to prevent further inflow of potassium chloride. There will always be enough potassium chloride between the stop-cock and its jacket to transmit the necessary E. M. F. In the writer's opinion the error is less and the convenience greater if the wicking is retained in the potassium chloride arm, and the outside stop-cock is left open in order to insure plenty of electrolyte along the whole course which the current must travel.

The apparatus is designed to meet the requirements of quick, accurate determinations in large numbers, and has been in use satisfactorily for a long period of time. A number of electrode parts B may be made from the same piece of platinum wire and, when treated in the same way as to blacking, may be substituted in the apparatus when necessary without loss of accuracy. All electrodes must of course be tested as to blacking against a standard acetate or other known buffer solution.

ON THE IDENTIFICATION OF CITRIC ACID IN THE TOMATO.

By R. E. KREMERS AND J. A. HALL.

(*From the Phytochemical Laboratory of Edward Kremers, University of Wisconsin, Madison.*)

(Received for publication, November 11, 1919.)

In spite of the large amount of work which has been done on the chemistry of the tomato, Hansen,¹ working in this laboratory a few years ago, was the first to isolate citric acid in crystal form from the juice of the tomato and to make an elementary analysis of his material. With more material available, it was desired to repeat Hansen's experiments. The recent work of Reid² and his collaborators suggested the application of derivatives of citric acid to its identification.

Citric Acid Triphenacyl Ester.—In order to familiarize ourselves with the procedure, the reaction was applied to U. S. P. sodium citrate. Instead of using the phenacyl bromide in slight excess, as Reid suggests, our experience seems to indicate that a slight excess of sodium citrate is preferable. The record of a typical experiment is as follows: 3.263 gm. of sodium citrate were dissolved in 27.5 cc. of water and 5.4114 gm. of phenacyl bromide and 55 cc. of 95 per cent alcohol were added. Alcohol has the property of precipitating an aqueous solution of Na citrate. On continued refluxing with frequent agitation the aqueous layer gradually disappears. The attainment of homogeneity is a good indication that the reaction is nearly complete. Refluxing on a boiling water bath was continued for 4 hours. The reaction mixture was poured into a crystallizing dish, set upon the water bath after the

¹ Unpublished results.

² Reid, E. E., *J. Am. Chem. Soc.*, 1917, xxxix, 124. Lyman, J. A., and Reid, E. E., *J. Am. Chem. Soc.*, 1917, xxxix, 701. Lyons, E., and Reid, E. E., *J. Am. Chem. Soc.*, 1917, xxxix, 1727. Rather, J. B., and Reid, E. E., *J. Am. Chem. Soc.*, 1919, xli, 75.

flame had been extinguished, and allowed to stand. If in a day or two the precipitate has not crystallized, it is redissolved in hot 95 per cent alcohol, from which it is usually obtained crystalline. Melting point 105° . Reid found 104° .

Unfortunately Reid gives no analytical data for his compounds. The following results were obtained with our material.

Combustion.—(No. 1.) 0.2159 gm. of ester gave 0.0975 gm. of H_2O and 0.5243 gm. of CO_2 .

(No. 2.) 0.2452 gm. of ester gave 0.1133 gm. of H_2O and 0.5907 gm. of CO_2 .

	Carbon. per cent	Hydrogen. per cent	Oxygen. per cent
No. 1.....	66.23	5.00	28.77
" 2.....	65.70	5.14	29.16
Theoretical.....	65.93	4.76	29.31

Saponification.—A quantitative saponification was undertaken several times; each time the results were too high for a tri-ester and no definite end-point could be located. Even cold $N/2$ NaOH gave too high results. According to Beilstein, phenacyl alcohol is readily decomposed even by dilute alkali.

Although Reid had obtained practically negative results in his attempts to prepare mono-*p*-nitrobenzyl esters of di-basic acids, it seemed worth while to try at least a few orienting experiments along the same line before proceeding with the acids from the tomato. Accordingly di- and monosodium citrate were prepared according to the directions of Heldt.³

The phenacyl esters were prepared in the same manner as the tri-ester; *i.e.*, the Na salt was slightly in excess of the theoretical quantity. The esters were crystallized as before.

	Di-ester. °C.	Mono-ester. °C.
M. P. of ester.....	104-105	104.5
M. P. mixed with tri-ester.....	105	104.5

Therefore the triphenacyl ester separates in each case. Perhaps the simplest explanation, in the absence of more experimental data, is that Heldt's mono- and disodium citrates are not truly named, but are merely mixtures of trisodium citrate and citric

³ Heldt, W., *Ann. Chem.*, 1843, xlvii, 157.

acid having the analytical composition indicated by their names. The method of preparation and the fact that Heldt prepared no corresponding salts by double decomposition with other metals rather support this supposition.

Application to the Tomato.

Material.—The material was obtained by saturating strained tomato pulp⁴ with calcium hydroxide; the Ca citrate is thereupon precipitated by boiling. That the material thus prepared is not nearly pure Ca citrate is shown by the analysis for Ca.

Found 15.6 per cent. Ca citrate requires 24.1 per cent.

The impure Ca salt did not yield crystals when brought into reaction with phenacyl bromide. Its purification was undertaken as follows after various trials:

The impure Ca salt was converted into the Na salt by boiling with Na_2CO_3 . Ca citrate was reprecipitated by adding NH_4Cl and CaCl_2 in excess to the solution of the Na salt, boiling and filtering while hot. The Ca salt dissolves in 50 per cent acetic acid on continuous stirring, from which solution the Pb salt is precipitated by basic Pb acetate. The Pb salt was decomposed by H_2S , the citric acid solution filtered from the Pb sulfide, concentrated *in vacuo*, and crystallized in a vacuum desiccator. The triphenacyl ester was then prepared as follows:

0.7382 gm. of citric acid dissolved in 9 cc. of water was neutralized with 0.5389 gm. of Na_2CO_3 . 1.61 gm. of phenacyl bromide dissolved in 18 cc. of alcohol were added and the mixture was refluxed for 3 hours. The reaction mixture was crystallized; yield 0.52 gm., melting point $104-105^\circ$. Mixed with ester from U. S. P. sodium citrate, melting point was $104-105^\circ$.

Therefore the presence of citric acid in the tomato juice has been shown by means of its triphenacyl ester.

⁴ For the material at our disposal we are much indebted to the Tomato Products Company of Paoli, Ind.

THE STRUCTURE OF YEAST NUCLEIC ACID.

V. AMMONIA HYDROLYSIS.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, December 1, 1919.)

In the preceding publication of this series¹ the statement was made that on mild hydrolysis with 5 per cent ammonia at a temperature of 100°C. yeast nucleic acid is broken up into four mononucleotides. The publication contained a report of the isolation of only three of these. At the time when that publication went to press, the fourth nucleotide had been isolated in the form of the brucine salt. However, we have learned that for the identification of a nucleotide one cannot depend on the analysis of only the brucine salt, when this salt is obtained from a mixture of brucine salts of several nucleotides.

The present publication contains a report on the isolation of the fourth nucleotide, the crystalline cytidinphosphoric acid. It also presents an example showing that not only the brucine salts, but also the free nucleotides are capable of forming mixed crystals, which may furnish analytical data very nearly approaching those required by polynucleotides. A crystalline substance was isolated, which, on the basis of its elementary analysis, could easily have been taken for an adenosin-uridin dinucleotide. On recrystallization from dilute alcohol, the substance was fractionated into cytidinphosphoric and adenosinphosphoric acids. Thus it is evident that the danger of error is extremely great if one assumes the existence of di- or trinucleotides on the basis of the results of elementary analysis.

EXPERIMENTAL.

The material for the present work was the brucine salts of the adenine fraction. It was stated in the preceding communication that these brucine salts were recrystallized nine times out of

¹ Levene, P. A., *J. Biol. Chem.*, 1919, xl, 415.

35 per cent alcohol. The final product consisted of the brucine salt of uridinphosphoric in a practically pure state. This was demonstrated by the fact that, when the brucine salt was converted into the ammonium salt, this without further purification was in a crystalline state and had an elementary composition required by the theory of that salt.

The first three mother liquors combined and concentrated yielded a brucine salt containing 10 per cent nitrogen, which served for the isolation of adenosinphosphoric acid described in the preceding publication.

The mother liquors from the fourth to the ninth (inclusive) recrystallizations served as starting material for the isolation of cytidinphosphoric acid. The crude brucine salts obtained on concentration of the mother liquors were first fractionated by means of methyl alcohol. For this purpose the brucine salts were suspended in methyl alcohol, boiled for 15 minutes on boiling water bath, and filtered while hot. There were obtained an insoluble part A and the mother liquor, which on cooling deposited a sediment B; the mother liquor from B on concentration under diminished pressure gave a third precipitate C. The last was too small for further work. Fraction A was recrystallized three times out of 35 per cent alcohol. The brucine salt had a nitrogen content $N = 7.71$ per cent. It was converted into the ammonium salt, and this into the lead salt. The latter was suspended in water. Through the suspension hydrogen sulfide gas was passed and the filtrate from lead sulfide was concentrated to small volume under diminished pressure, finally placed in vacuum desiccator over sulfuric acid, and allowed to crystallize under diminished pressure. When the solution was concentrated to the consistency of a syrup, there began to form a crystalline deposit consisting of long needles. The mother liquor was very viscous and the crystals were freed from it by repeated washing with hot methyl alcohol. Apparently the mother liquor contained some uridinphosphoric, which is extremely soluble in water.

The crystalline substance was analyzed without recrystallization. It had a melting point M.P. = 225°C . (corrected) with decomposition. The analysis of the substance was as follows.

0.1106 gm. of the substance employed for Kjeldahl nitrogen estimation required 10.34 cc. of 0.1 N acid.

	Calculated for $C_5H_{11}N_3PO_7$, per cent	Found. per cent
N.....	13.00	13.09

The optical rotation of the substance was as follows.

$$[\alpha]_D^{25} = \frac{+1.05 \times 100}{1 \times 2} = +52.5$$

The rotation of the cytidinphosphoric acid isolated by Thannhauser was $[\alpha]_D^{25} = +23.3$, and the value calculated from the barium salt described by the writer was also $[\alpha]_D^{25} = +23$. The reason for this discrepancy will have to be established. The two latter preparations were obtained on acid hydrolysis.

Fraction B was recrystallized four times out of 35 per cent alcohol. The resulting substance was converted into the ammonium salt, which in its turn was converted into the lead salt, and this was freed from lead and thus a solution of free nucleotides was obtained. This was concentrated under diminished pressure and the solution allowed to crystallize in the open air. Crystallization began rapidly and was completed after 3 days. The crystal form differed from that of the pure nucleotides. The latter appear in form of long fine needles, while these had the appearance of heavy prisms. The substance was recrystallized once out of hot water and then had the following composition.

0.1170 gm. of the substance gave 0.1453 gm. of CO_2 and 0.0419 gm. of H_2O .

0.1930 gm. of the substance employed for Kjeldahl nitrogen estimation required 20.60 cc. of 0.1 N acid for neutralization.

0.2845 gm. of the substance gave 0.0954 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{19}H_{28}N_7O_{14}P_5$, per cent	Found. per cent
C.....	34.90	33.86
H.....	3.86	4.12
N.....	15.01	14.94
P.....	9.50	9.19

The optical rotation of this substance was

$$[\alpha]_D^{25} = \frac{+0.40 \times 100}{1 \times 2} = +20.0$$

Thus the substance could easily be taken for a dinucleotide. It was separated into two fractions in the following manner: 4.8 gm. of the substance were dissolved in 150 cc. of hot water, and to this solution 300 cc. of 99.5 per cent alcohol were added gradually. On cooling a crystalline deposit began to form. After 1 hour this was removed by filtration. The yield of the air-dry material was 2.8 gm. (Fraction A). In the mother liquor on concentration under diminished pressure a deposit formed consisting of long needles (Fraction B).

Fraction A₁ analyzed as follows.

0.1006 gm. of the substance gave 0.1216 gm. of CO₂ and 0.040 gm. of H₂O.
0.2000 " " " " employed for Kjeldahl nitrogen estimation
required 18.92 cc. of 0.1 N acid.

0.2940 gm. of the substance gave 0.0982 gm. of Mg₃P₂O₇.

The optical rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.80 \times 100}{1 \times 2} = +40.0$$

Once recrystallized out of water the substance analyzed as follows.

0.0986 gm. of the substance gave 0.1206 gm. of CO₂ and 0.041 gm. of H₂O.

0.1973 gm. of the substance employed for Kjeldahl nitrogen estimation
required for neutralization 18.33 cc. of 0.1 N acid.

The optical rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.86 \times 100}{1 \times 2} = +43$$

	Calculated for C ₅ H ₁₀ N ₂ PO ₃ per cent	Sample A ₁ . per cent	Found. Sample A ₂ . per cent
C.....	33.42	33.57	33.35
H.....	4.37	4.44	4.64
N.....	13.00	13.51	13.01
P.....	9.61	9.30	

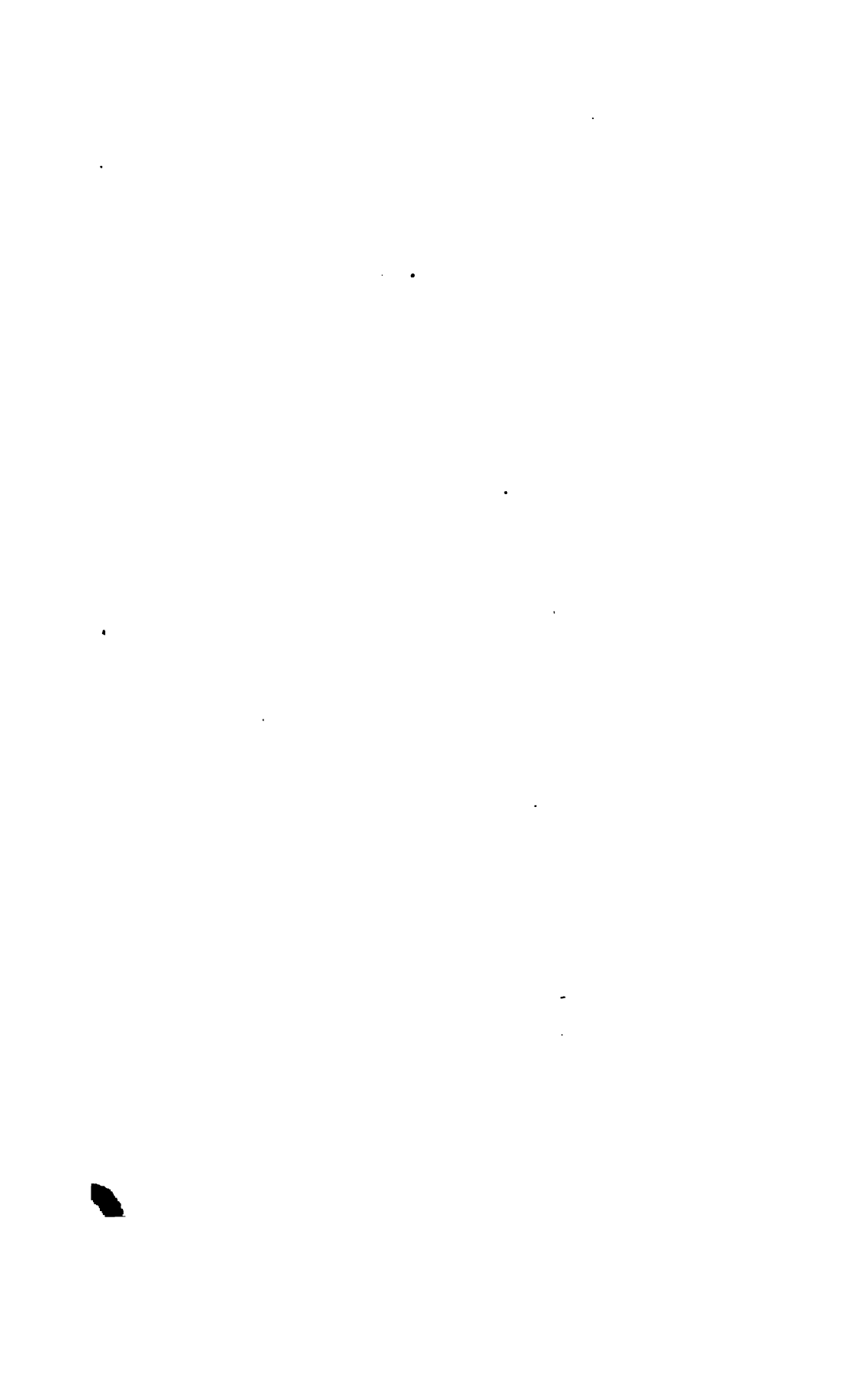
Fraction B₁ analyzed as follows.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation
required for neutralization 26.24 cc. of 0.1 N acid.

	Calculated for $C_{18}H_{34}NaPO_7$ per cent	Found. per cent
N.....	20.17	18.44

The optical rotation of the substance was

$$[\alpha]_D^{25} = \frac{-0.78 \times 100}{1 \times 2} = -39.0$$



QUANTITATIVE ESTIMATION OF INDOLE IN BIOLOGICAL MEDIA.*

By HARPER F. ZOLLER.

(From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture, Washington.)

(Received for publication, November 25, 1919.)

The demand for a rapid and yet delicate method for quantitatively estimating indole arose from a study of its progressive formation in certain bacterial cultures in this laboratory. It is rather remarkable that few methods have been proposed for determining this important product of the metabolism of certain microorganisms. Among those deserving attention are the suggestions of Herter and Foster (1), for the successful application of which we are indebted to Gorter and de Graff (2), and more especially to Bergeim (3); also the application of the nitroso reaction by von Moraczewskii (4) and by Nonnotte and Demanche (5). Both these methods depend upon the use of very sensitive color reactions.

The use of the color reaction of Ehrlich (*p*-dimethylamino-benzaldehyde) was considered but dismissed after a few trials for several reasons: (a) it reacts readily with phenolic compounds; (b) it is extremely slow in attaining equilibrium as regards its full deep color (6); (c) it is a difficult compound to obtain, and was expensive at the time of the investigation; and (d) the reagent deteriorates rapidly.

The method as devised by Bergeim, employing the very sensitive color reaction of Herter and Foster (beta-naphtha-quinone-sodium-monosulfonate), is fairly dependable and sensitive, but is objectionable in several respects. In the first place the reaction cannot be used in the presence of phenols or ammonia, which necessitates the separation of the indole from these substances by distillation. Solutions of beta-naphtha-quinone-sodium-mono-

* Published with the permission of the Secretary of Agriculture.

sulfonate deteriorate rapidly, the compound being subject to ready oxidation. At present it is difficult to obtain the reagent upon the open market, although this objection is hardly valid, since the compound could be produced if the demand were prevalent. A more serious objection, and one common to all methods involving the use of the colorimeter, is the question of the expense of the instrument. Many laboratories do not possess a standard colorimeter.

As proposed, the method of Bergeim requires the cumbersome and laborious process of steam distillation for the separation of the indole from the fecal suspensions. Since many substances seem to interfere with the accuracy of the determination it is necessary to repeat the steam distillation process, in the first instance using an alkaline medium and afterwards an acid one. Bergeim points out the use of Folin and Bell's exchange silicate (*permutit*) (7) for the absorption of ammonia, and his results seem to show that it is possible by its use to evade one of the distillations, although the employment of *permutit* following the first steam distillation requires considerable manipulation. In my own experience it has been found possible to remove small quantities of several of the alkylamines with exchange silicate, notably monomethyl- and monoethyl- and dimethyl-amine.

In 1908 Nonnotte and Demanche (5) published a method which proffered the possibilities of being developed into one possessing great simplicity as well as accuracy. They made use of the familiar nitrite color reaction of Baeyer (8) and Nencki (9), which involves the addition of a dilute nitrite solution to an aqueous solution of indole, and following this with a few drops of hydrochloric, sulfuric, or nitric acid. Nonnotte and Demanche specify ten drops of 1 per cent nitrite and four drops of concentrated sulfuric acid to 10 cc. of the centrifuged medium. The intensity of the color, after a given time limit, is compared to a series of standard tubes containing known quantities of indole treated as above.

Hopkins and Cole in 1903 (10) and Herter (11) in 1908 made extensive study of the chromogen of the "urorosein" urine reaction and discovered that indoleacetic acid as well as indole could be responsible for the red color produced when urines containing nitrites were treated with HCl or H₂SO₄. Herter also found that

p-dimethyl-amino-benzaldehyde reacted with indoleacetic acid to give a coloration similar to that with indole. With this in mind it is obvious that Nonnotte and Demanche's use of the nitroso reaction in cultures, freshly centrifuged to relieve turbidity, is fraught with grave danger, since indoleacetic acid (skatole carbonic acid, Salkowski (12)) is reported to have been isolated from cultures of *Bacillus coli* by Hopkins and Cole (10) and putrefactive mixtures by Salkowski (12). Homer (6), in a recent and very exhaustive study of the chromogen of the urochrome and allied reactions, has more clearly identified the precursors of these color changes. For most purposes it should be conceded that, in order to render even the detection of indole in bacterial cultures and other media at all sensitive and reliable, it is necessary to apply the test to the distillate after proper distillation. The limitations for a proper distillation formed the objective of a separate study and the results are reported in another paper (13).

Another fact which was evidently lost sight of by Nonnotte and Demanche is the solubility of nitroso-indole in weakly acid solutions. When a solution, containing a concentration of indole greater than 0.15 mg. per 10.0 cc. of solution, is treated with nitrite and acid the red nitroso-indole compound supersaturates the solution and gradually settles on the side of the container. The discovery that the sensitivity of the nitroso reaction with indole could be increased more than threefold by resorting to a few cc. of either isoamyl (primary) or isobutyl (primary) alcohol at once suggested the value of the test for detection. The alcohol completely extracts the nitroso-indole and rises to the surface, thereby concentrating to a small area the full amount of color. This discovery, together with the result that these solutions of nitroso-indole possess great permanence over long periods of time, led us to believe that the method of Nonnotte and Demanche could be so improved as to furnish one of reliable application.

Separation of Indole from Complex Mixtures.

Technique.—As reported in another study (13) it was found that the method of steam distillation could be abandoned for the more available one of direct distillation, provided recognition was given to the tendency of indole to volatilize more readily from solutions

made alkaline above a certain hydrogen ion concentration and within the limit of no hydrolytic action. The particular range of hydrogen ion concentration which gave complete volatilization of the indole from the distilling flask when 75 per cent of the contents had been driven over, and which showed no indication of loss of indole through destructive action, was from pH 8.5 to 10.5; the highest alkalinity studied was pH 10.5. The point arbitrarily chosen to which all solutions should be adjusted before distilling is pH 9.2. This point was taken in the first place because of the ease of adjusting the reaction of the solution to this intensity of acidity. At pH 9.2 phenol- or cresolphthalein¹ are both at their medium of intensity of clear red-violet color, while thymolphthalein fails to show a visible blue color (the first appearance of color with thymolphthalein is between pH 9.3 to 9.4). In the second place the degree of foaming increases with protein solutions as the alkalinity increases, while at the point chosen very little trouble was experienced. Furthermore, higher alkalinities tend toward harsh hydrolysis of protein material and liberate quantities of ammonia. While small amounts of ammonia were not found to hamper the delicacy of the test in any way, large amounts may just as well be avoided.

The frothing in alkaline solutions may be checked in a large measure by the addition of various compounds, but in the case of my own experience a little care exercised during the distillation was all that was needed. One precaution that was uniformly taken was to seal one end of a glass tube about 6 to 8 mm. in diameter and insert it into the distilling flask with the open end on the bottom of the flask beneath the surface of the liquid and the opposite end resting against the neck of the flask so that it would remain in this position throughout the distillation. A constant flow of vapor is maintained by this form of ebullator and the tendency to foam is diminished. Many of the high boiling point compounds used to reduce foaming are solvents for indole and thereby tend to concentrate it in different regions within the distilling flask. Phenylether as proposed by Mitchell

¹ The color transitions of phenolphthalein and *o*-cresolphthalein occur in approximately the same zone of hydrogen ion concentration. The latter indicator may be used more advantageously in deeply colored media since its color changes are more brilliant than those of phenolphthalein.

and Eckstein (14) gives as satisfactory results as any in solutions where the tendency to foam is abnormally great.

A satisfactory procedure when working with bacterial cultures is to grow them in Florence Pyrex flasks of 300 cc. capacity, employing 100 cc. of medium per flask. Connect the flask with an upright water-cooled condenser so that the discharge of the condensate is rapid and complete. A rubber stopper covered with tin-foil provides a clean and vapor-tight connection between flask and condenser.

Adjustment of Reaction.—The culture or medium containing indole is adjusted by the addition of normal NaOH to the desired degree of hydrogen ion concentration as shown by phenolphthalein and checked by thymolphthalein. This may be accomplished, *where extreme accuracy is desired*, by pipetting 10 cc. of the medium into a test-tube, adding five drops of the 0.02 per cent indicator solution, and then adding alkali drop by drop until the depth of color matches the depth of color produced by the same quantity of the indicator in 10 cc. of a standard buffer mixture. This standard buffer mixture may be prepared to represent a true hydrogen ion concentration of pH 9.2, or any other decided upon for the distillation, by following the method proposed by Clark and Lubs (15). If the 10 cc. of medium represent one-tenth of the total volume to be distilled, then by multiplying the number of drops or cc. of alkali required for the one-tenth portion by nine, the quantity of alkali necessary for the remaining 90 cc. to reach the same pH will be determined. A burette is convenient for holding and measuring the alkali. The 10 cc. portion containing the indicator and alkali may be returned to the flask for distillation.

If no standard buffer mixture is at hand it is possible to adjust the reaction directly in the flasks, and *this is to be recommended in all cases of laboratory routine and when working with pathogenic organisms*. All that is necessary in this case is to run normal alkali into the solution until phenolphthalein shows a full red and thymolphthalein shows no blue. One drop of indicator as it falls on the surface of the medium is sufficient to indicate these pH values when observed at the point of contact. The writer has successfully used a porcelain-spot test-plate for determining the reaction. Two or more drops of media and one drop of the 0.02 per cent indicator solution give excellent definition.

The distillation is continued until all but about 10 cc. has been driven over, the distillate being collected in a 100 cc. volumetric flask and made up to the mark. This represents the total indole from the culture.

Determination of Indole.

Preparation of Standards.—10 mg. of pure indole are dissolved in 100 cc. of distilled water, warming the water slightly if necessary. A set of 10 test-tubes $\frac{1}{2}$ inch in diameter and of uniform thickness and color are arranged and numbered to receive portions of the above indole standard delivered from a burette graduated in 0.1 cc. (0.01 cc. if obtainable). It has been found useful to arrange the following concentrations in the tubes: 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, and 0.20 mg. of indole respectively. Water is now added to the tubes to approximate a total of 10 cc. To each tube are added two drops of a 1.0 per cent solution of sodium nitrite (NaNO_2) and five drops of concentrated sulfuric acid. Each tube is carefully shaken by inversion. After standing for about 5 minutes each tube is extracted with three portions (3 cc. per portion) of isoamyl or isobutyl alcohol. These three extractions are combined in one test-tube and made up to exactly 10 cc. with the pure extractant; this represents the total nitroso-indole from the respective indole standards. Both isoamyl and isobutyl alcohols, under the conditions of the test, form clear strata above the water portion without emulsification and can be drawn off by means of a pipette, the stem of which is bent at right angle and possesses a soft rubber tube. The test-tubes can be marked at the 10 cc. volume point.

These alcoholic nitroso-indole standards are thoroughly reliable for a working period of a week or longer. They may be placed in subdued light when not in use. They have been observed for a period of 2 months in strong light and the only change observed was a slightly increased detail of the absorption bands. No new bands were noticed; neither was there shifting of the old. No change was apparent to the eye.

Details of Test.—An aliquot of the distillate from the indole separation is pipetted into a test-tube of convenient size; the aliquot may be 10, 15, or 20 cc., or larger, just so that no more

than 0.20 mg. of indole is present in it. If the quantity of indole is known to be small then a larger aliquot is necessary, since there must be at least 0.01 mg. in order to compare with the above mentioned standards. Of course any similar set of indole standards may be made up, employing different concentrations. For instance Nonnotte and Demanche advised the use of the drop system in measuring the indole solutions for the nitroso-indole standards. This latter system is obviously very faulty, and was found to yield varying results.

The aliquot of distillate is measured into a test-tube and two drops of 1.0 per cent NaNO_2 solution are then added, this being followed by five drops of concentrated sulfuric acid. The test-tube is shaken and allowed to stand about 5 minutes for the nitroso reaction to approach equilibrium. It is then extracted with three portions (3 cc. per portion) of isobutyl or isoamyl alcohol as before, and each portion is drawn off with a pipette and run into a test-tube of the same dimensions and quality as those which hold the standards. This tube should previously be marked at the 10.0 cc. volume point, so that after adding the last portion of extraction it may be made up to the mark and compared with the standards. It is advisable to run duplicates on the distillates at the same time. With a little experience it is possible to interpolate very accurately between successive 0.02 mg. standards by matching the relative intensity.

If it is desired to work with smaller portions of cultures or biological products the same procedure as outlined above may be followed, with the added precaution of diluting the mixture or culture to 100 cc. before distilling. In this case it may be necessary to work with the entire distillate, when a small separatory funnel will be found convenient.

*Comparison of the Nitroso-Indole Method with the Beta-Naphtha-
Quinone-Sodium-Monosulfonate Method as Applied
by Bergeim.*

2.5 mg. of Eimer and Amend indole were dissolved in 200 cc. of distilled water. This was used as standard for the comparative determinations, portions of which were carefully measured into tubes from which the contained indole was extracted. Chloro-

form, as suggested by Bergeim, was used as an extractant when following his method. Isobutyl (primary) alcohol was used to extract the nitroso-indole. A 1.0 cc. solution of indole containing 0.10 mg. was diluted, treated with beta-naphtha-quinone-sodium-monosulfonate² and extracted with chloroform, and the total chloroformic extract was made up to 15 cc. This was used as

TABLE I.

Description of experiment.	Indole.			
	Bergeim method.		Zoller method.	
	In portion.	Found.	In portion.	Found.
	mg.	mg.	mg.	mg.
15 cc. portions of prepared indole solution	0.1375	{0.1362 0.1365	0.1375	{0.1350 0.1350
10 cc. portions of prepared indole solution	0.1250	{0.1246 0.1248	0.1250	{0.1300 0.1250
200 cc. culture of <i>B. coli</i> grown on medium for 30 hrs.; 100 cc. by Bergeim method, 100 cc. by Zoller method*		{0.465 0.468		{0.478 0.476
10 cc. portion of preparation + 1.0 per cent of phenol			0.1250	{0.1275 0.1250
10 cc. portion + 1.0 cc. of 0.1 N NH ₃ .			0.1250	{0.1300 0.1250

* Time required for Bergeim's separation and test, 3.5 hrs.; for Zoller's separation and test, 1.0 hr.

standard in a Duboscq colorimeter for all determinations which were based upon Bergeim's method.

As a further test of the practicability of the proposed method for estimating indole, a culture of *Bacillus coli* (Lab. No. fg) was grown in 500 cc. of a medium of 1.0 per cent Difco peptone, 0.5

² The writer uses this opportunity to thank Dr. O. Bergeim, of the Jefferson Medical College, Philadelphia, Pa., for a liberal quantity of beta-naphtha-quinone-sodium-monosulfonate furnished for indole determinations.

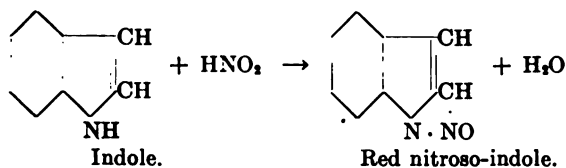
per cent K_2HPO_4 , and 0.1 per cent dextrose for 30 hours. Two portions of 100 cc. each were simultaneously removed from this culture, and the indole was removed from one portion by Bergeim's double distillation method, and from the other portion by the scheme involving adjustment of reaction and distillation as outlined in this paper. The indole was determined in portions of the separate distillates and calculated to milligrams of total indole in 100 cc. of the culture. The results are presented in Table I.

DISCUSSION.

It should be remembered that skatole is not an infringing danger to the success of the proposed method. Fischer (16) and Brieger (17) as well as Salkowski pointed out that nitroso-skatole, if formed, was white and insoluble in the acid solution in which it is formed. The writer has found it to be only slightly soluble in isobutyl alcohol, and it did not give absorption in the visible portion of the spectrum. It seems likely therefore that skatole would not interfere in the determination.

Indoleacetic acid, as previously mentioned, reacts similarly with nitrous acid to give a red nitroso-indoleacetic acid. This may be responsible for the "cholera-red" reaction of Nencki (18) as well as the urosein. Since indoleacetic acid is non-volatile with water vapor it is eliminated as a danger to the accuracy of the test.

It is very likely that the formation of nitroso-indole follows the reaction



Nitroso-indole is readily soluble in methyl alcohol and can be crystallized from it in flesh-colored, shiny leaves or flakes. These seem to remain unchanged for long periods of time and suggest the possibility of preparing standards directly from the purified crystals. The crystals melt at 170°C . (uncorrected), with decomposition.

The nitroso-indole reaction, when supplemented with the alcohol extraction, becomes an exceedingly sensitive and convenient way of detecting the presence of indole. The relative sensitivity of Herter's naphthaquinone reaction and the nitroso reaction were found to be respectively 1:2,000,000 and 1:1,500,000. The extraction of the excess of the reagent in the former case by the chloroform reduces noticeably its sensitiveness. It is also necessary that the naphthaquinone reagent be freshly prepared in order to secure best results. With the nitroso-indole test the only precautions necessary are the proper concentrations of nitrite solution and acid. Excess nitrite or acid tends to displace the sensitiveness. The most effective working limits for the reaction as applied seem to be those given in the above method. It is important to know that the alcoholic solutions of nitroso-indole are much more stable than the aqueous solutions.

Recently a number of investigators have used the vanillin test proposed by Steensma (19). Blumenthal (20) reported in 1909 a very careful survey of the color reaction between various aromatic aldehydes, including vanillin, and indole and skatole. While many of these aldehydes yield beautiful color reactions with indole and skatole, their sensitiveness is greatly impaired because of the high reactivity of the aldehydes for other compounds such as phenols and ammonia. The danger of performing the vanillin test in cultures of organisms is at once apparent. The yellow-orange color which develops when indole and vanillin are brought together in acid solution is very readily masked, as well as duplicated by other substances. When the test is applied to the indole distillate from such cultures its delicacy is only slightly more enhanced and even then is not so discriminating between the homologues as the nitroso-indole, *p*-dimethyl-amino-benzaldehyde, or naphtha-quinone-monosulfonate reactions. The arguments brought forward for its extreme delicacy are mainly fallacious in view of these facts. The writer, therefore, believes that a note of warning should be sounded against its promiscuous use in bacteriological work.

The rate at which the equilibrium of the nitroso-indole reaction is reached depends upon the factors of concentration and temperature. It is notable that with temperatures above normal, 30 to 90°C., the full depth of color is obtained in a few seconds. Higher

temperatures tend to destroy the colored chromogen when in aqueous solutions. In pure isobutyl alcohol the color is not destroyed even after several hours' heating at temperatures around 100°C. Inasmuch as indole is volatile with water vapor, the rate being chiefly dependent upon temperatures, it is unwise to conduct the test or determination at temperatures above 30°C.

It seems that the features of readily available reagents and simplicity and speed of performance are in themselves sufficient to recommend the proposed method to any one. In addition to these points it is well to consider the fact that indole occurs in biological media in the extremes of concentrations and that the foregoing method as devised is particularly adapted to measure these extremes. Since the evidence reported shows it to check favorably with the best from the standpoint of accuracy and its sensitiveness to be less affected by contaminating substances, it should find very general application.

SUMMARY.

1. The limitations of the nitroso-indole reaction of Baeyer have been studied with the idea of improving and standardizing the Nonnotte and Demanche application of it to the quantitative determination of indole.

2. The method evolved is simple, reliable, and rapid, and requires only the reagents and apparatus common to most laboratories.

3. The indole is separated from the parent mass by one direct distillation. The limitations of this distillation have been reported elsewhere (13) and are dependent upon the hydrogen ion concentration of the medium.

4. Use of the nitroso reaction, when accompanied by an alcohol solvent, for testing the distillate from biological media for the presence of indole is advocated. A warning is sounded against the use of the vanillin-acid test for indole.

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INFLUENCE OF HYDROGEN ION CONCENTRATION UPON THE VOLATILITY OF INDOLE FROM AQUEOUS SOLUTION.*

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The methods in common practice for the separation of indole from biological mixtures stipulate the addition of either acid or alkali of strong concentration to the flasks of suspended material from which the indole is to be driven by steam distillation or direct boiling. The purpose of such treatment is, doubtless, to prevent the volatilization of interfering substances by neutralizing them *in situ*. Not infrequently one finds the direction to double distill; *i.e.*, distill first from alkali with steam and then acidify the distillate and redistill with steam or *vice versa*. In the procedures published in the literature no apparent thought is given to the final reaction of the medium, or to the reserve alkalinity or acidity which it possesses. No attempt is made to identify the concentration of the acid or alkali with its chance effect upon the indole molecule, either internally or externally. It is the purpose of this paper, therefore, to report the results of a study aimed toward the defining of standard conditions for the separation of indole from various media. In projecting the study two objectives were sought. One was to determine whether steam distillation could be safely abandoned for direct distillation. The other was to ascertain whether or not the conditions for such a separation would destroy the indole molecule.

Since indole is an aromatic imine possessing basic properties—although the degree of basicity is greatly modified by the orien-

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tation within the double-ring structure (as proved by the catalytic reduction of indole into *o*-toluidine and methane)—(1) we should expect it to form associated compounds with strong acids. In this expectation we are not disappointed, for the compounds indole nitrate (2) and indole hydrochloride (3) have been isolated. The methods of isolation and their properties suggest that these compounds are very loosely associated. The fact that indole may be removed from a strong acid solution by steam, when proper cautions are observed, tends to justify this assumption.

As previously mentioned, the concentration of acid employed in the isolation of indole has been rather high, although varying through a wide range. It is well known that a hydrogen ion concentration maintained at 1.0×10^{-8} is amply sufficient to prevent the volatilization of ammonia from aqueous solutions. In many instances in the practice of distilling indole with water vapor the hydrogen ion concentration is in excess of 1.0×10^{-1} . Now it was thought that the rate at which indole volatilized from aqueous solutions, adjusted to definite initial hydrogen ion concentration, might be of value in defining the conditions under which the distillation of indole could be safely conducted.

One could anticipate, inasmuch as indole possesses feebly basic properties, that it would volatilize more freely from an alkaline solution. To what extent the alkalinity might be carried without producing disintegration of the indole molecule has never been, to the writer's knowledge, published. This might be roughly ascertained by determining the indole distilled and that remaining in the distillation flasks after a series of diverging alkalinities had been run. This, of course, would necessitate the assumption that the products resulting from the action of alkali on indole do not act like indole toward the test reagents. Such an assumption is only partially justifiable. In very strong alkali solutions indole converts first to indoxyl and thence to indigo blue, following the course of a true oxidation. Neither of these substances would confuse the nitroso test for indole.

With these considerations in mind and with a rather rapid method of determining indole at hand (4), the following study was made.

Experimental A.

Twelve Florence-shaped, 250 cc., Pyrex flasks were provided. Into each were poured 90 cc. of distilled water containing 1.0 mg. of indole. The hydrogen ion concentration of each solution was initially adjusted to the respective pH values, the term $\text{pH} = \log \frac{1}{\text{hydrogen ion}}$ being that adopted by Sørensen (5).

Flask No.	pH	Indicator.	Flask No.	pH	Indicator.
A	1.0	Thymol blue.	G	6.0	Bromocresol purple.
B	2.0	" "	H	7.0	Phenol red.
C	3.0	Bromophenol blue.	I	7.6	" "
D	4.0	" "	J	8.0	Cresol "
E	4.4	Methyl red.	K	9.0	Cresolphthalein.
F	5.0	" "	L	10.0	Thymolphthalein.
			M	10.5	"

In adjusting the reaction of the solutions use was made of a 0.1 *N* solution of HCl and 0.1 *N* disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) for the range of pH 1.0 to 8.0, while for the range of pH 9.0 to 10.5 a 0.1 *N* NaOH solution in conjunction with the Na_2HPO_4 solution. Burettes carried the solutions mentioned. The indicators developed and reported by Clark and Lubs (6) were employed for accurately determining the initial pH points. In addition, thymolphthalein served to determine the concentrations pH 10.0 and 10.5. The total volume of solution within the flasks was finally adjusted to 100 cc. with distilled water. They were then placed upon an iron wire gauze over a free flame of a Bunsen burner and the contents directly distilled through glass connections into an upright, short tube (water jacket 7.0 inches) condenser. The condensate was collected in 50, 25, and 15 cc. portions, and in the noted order, graduated cylinders acting as collectors. The quantity of indole in each portion was determined by the nitroso-indole method (4). The amount of indole remaining in the flasks was also determined in the same manner.

It should be mentioned that a glass tube ebullator was placed in each flask before distilling in order to insure a constant flow of vapor into the condenser. The more alkaline solutions, pH 9.0 to 10.5, were carefully watched to prevent foaming. It was undesirable in this study to add chemical foam reducers since the majority of them (amyl, octyl, and heptyl alcohol and phenyl ether) are solvents for indole and immiscible with water; hence their use would introduce the question of effect upon the volatility of indole.

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The results of the experiment above are included in Table I. The values in the last column, expressing the per cent of volatility of indole from aqueous solution at different pH values are shown graphically in Fig. 1.

TABLE I.

Reaction of solution.	Indole in aqueous solution.					
	Flask.	50 cc.	25 cc.	15 cc.	Flask.	Distilling in first 75 cc.
pH	mg.	mg.	mg.	mg.	mg.	per cent
1.0	1.000	0.610	0.205	0.13	0.050	81.5
2.0	1.000	0.590	0.200	0.140	0.065	79.0
3.0	1.000	0.605	0.220	0.125	0.050	82.5
4.0	1.000	0.570	0.205	0.140	0.090	77.5
4.4	1.000	0.585	0.190	0.125	0.100	77.5
5.0	1.000	0.585	0.210	0.105	0.095	79.5
6.0	1.000	0.690	0.205	0.080	0.030	89.5
7.0	1.000	0.785	0.185	0.025	0.000	97.0
7.6	1.000	0.855	0.130	0.015	0.000	98.5
8.0	1.000	0.860	0.135	0.010	0.000	99.5
9.0	1.000	0.906	0.090	0.000	0.000	99.6
10.0	1.000	0.915	0.086	0.000	0.000	100.1
10.5	1.000	0.912	0.084	0.000	0.000	99.6

Since it might be construed that the above values would not be approached in complex media, although such media possessed corresponding pH values, it was, therefore, desirable to repeat the above experiments, employing a protein suspension in lieu of the aqueous solutions of indole. Consequently a medium was prepared which possessed approximately the following compositions: 2.0 per cent Difco peptone, 0.1 per cent dextrose, and 1.0 per cent K_2HPO_4 .

50 cc. of this medium were measured into each of the above flasks. 1.0 mg. of indole was added in the form of 10 cc. of a 0.1 per cent solution. The corresponding pH values were adjusted in the various flasks, eliminating pH 4.4 and 7.6. The final volume of solution was made up to 100 cc. and distilled as in the previous study. Flasks holding solutions at pH 9.0, 10.0, and 10.5 received 1.0 cc. of phenylether each to prevent excessive foaming. The results are reported in Table II.

TABLE II.

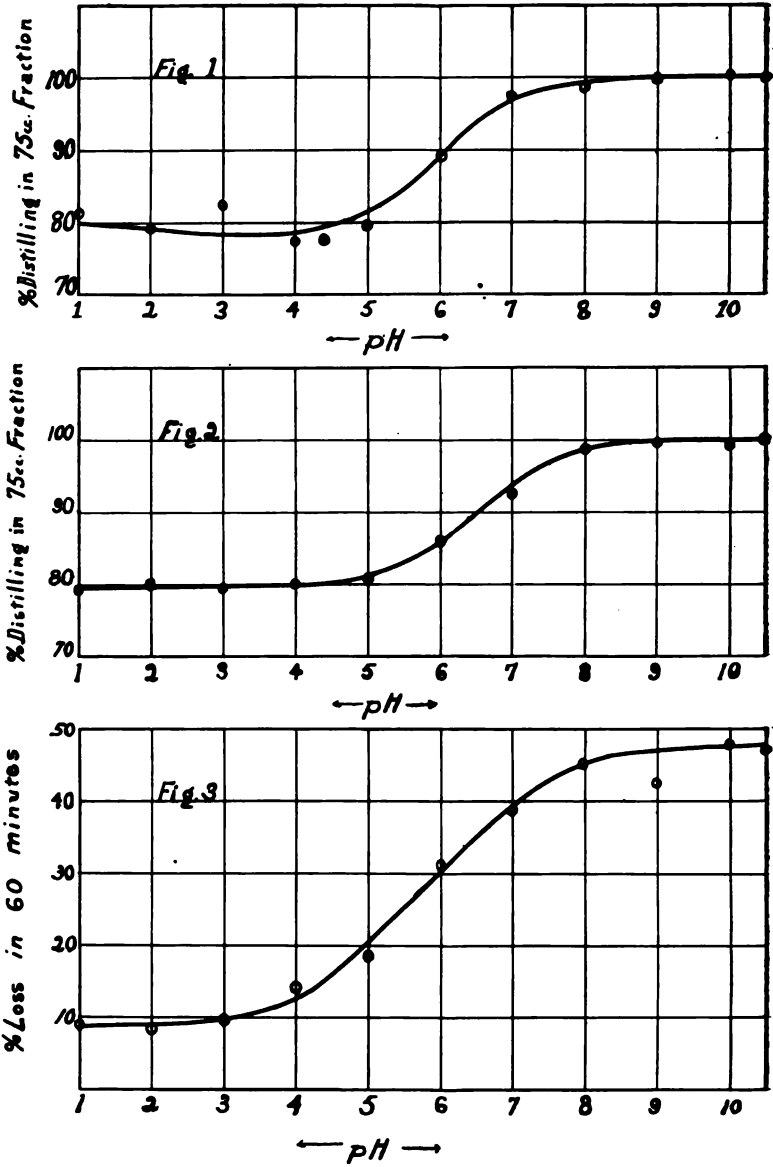
Reaction of suspension.	Indole in protein suspension.			
	Flasks.	50 cc.	25 cc.	Distilling in first 75 cc.
<i>pH</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1.0	1.000	0.585	0.205	79.0
2.0	1.000	0.600	0.200	80.0
3.0	1.000	0.595	0.210	80.5
4.0	1.000	0.595	0.205	80.0
5.0	1.000	0.590	0.220	81.0
6.0	1.000	0.620	0.240	86.0
7.0	1.000	0.725	0.200	92.5
8.0	1.000	0.875	0.115	99.0
9.0	1.000	0.890	0.105	99.5
10.0	1.000	0.910	0.080	99.0
10.5	1.000	0.905	0.095	100.0

No attempt was made herein to determine the distribution of indole beyond the first 50 and 25 cc. portion of distillate. It was assumed that no greater destruction of indole would occur in the mixed medium than in the purer solutions. Considerable ammonia was evolved from the last two flasks. The rate of volatilization of the indole was plotted against the pH and is reproduced in Fig. 2.

Experimental B.

In order to further establish the rate of volatility another set of aqueous solutions of indole was prepared according to methods followed under A, except that 2.0 mg. of indole were dissolved in a total volume of 200 cc. The range of pH was, with the exception of 4.4 and 7.6, the same as in Table I. Each solution of indole was placed in a gas-washing bottle possessing gas-tight junctures. This bottle was in turn set into a water bath (the water bath was maintained at 50°C. throughout the experiment) and then connected to a train of three other bottles B, C, and D. Bottle B contained distilled water, C 50 per cent NaOH, and D an improvised glass gas meter so constructed that 20 cc. of air were passed at each dump. Communication was now established between the bottle carrying the indole solution and the vacuum line, and the rate of suction so adjusted that 1,000 cc. of CO₂-free air passed through the indole solution each minute for 60 minutes.

After aerating each solution for 1 hour they were again adjusted to 200 cc. and the indole content was determined in an aliquot. The per cent of loss of indole is plotted against the pH and is represented in Fig. 3.



DISCUSSION.

It is clear from the published results that the tendency is towards an increased volatility of indole from solutions possessing a hydrogen ion concentration of 1.0×10^{-8} and less; or in terms of Sørensen's nomenclature, pH 8.0 to 10.5. There was no reason for the studying of acidities or alkalinities of a higher order, since the ranges of those investigated are extreme enough for all practical purposes.

The comparative regularity of the distillations as performed, and the completeness of the volatilization of the indole in the first 75 cc. of distillate from aqueous solutions possessing an initial pH of 8.0 and higher, suggest that the practice of steam distillation is unnecessary. A mixture containing indole and adjusted to a pH of 9.0 to 10.0 will not volatilize any phenols, but will permit some ammonia, skatole, and other volatile bases to be driven over with water vapor. Ammonia may be removed by shaking out with exchange silicate (permutit) or yellow mercuric oxide (7), while permutit will also remove small amounts of certain alkylamines. Neither of these absorbents will remove indole or skatole. A further test pointing to the regularity of the distillation was conducted by distilling 100 cc. of a solution containing 1.0 mg. of indole, buffered at an initial pH of 9.0, from a Duclaux still patterned after that designed by Gillespie and Walters (8). Nine successive 10 cc. portions of the distillate were collected and separately analyzed for indole. The ninth fraction was free from indole while the eighth contained less than 0.01 mg.

The application of this principle of direct distillation of indole from crude mixtures has been practiced in connection with some bacteriological work in this laboratory and with satisfying results. The method as applied is discussed in another paper (4). It may be remarked that the method is much preferable to the cumbersome one of steam distillation, especially when dealing with pathogenic organisms.

SUMMARY.

1. The range of most rapid volatilization of indole from the aqueous solutions studied is from pH 8.0 to 10.5.

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2. Hydrogen ion concentrations greater than 1.0×10^{-6} caused a decrease in volatility of indole with water vapor, probably due to the formation of weakly associated combinations between indole and acid in aqueous solution.

3. Hydrogen ion concentrations of the order of pH 10.5 have no appreciable destructive action on the indole molecule under the conditions of the study.

4. The results from the experiments performed suggest that the practice of steam distillation can be supplanted by direct distillation with equal accuracy when the reaction of the solution is taken into account. This direct method of distillation has been practiced in a routine investigation in this laboratory and found reliable and commendable.

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AVAILABILITY OF CARBOHYDRATE IN CERTAIN VEGETABLES.

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The extensive use of certain vegetables of low carbohydrate content, such as spinach, cabbage, cauliflower, and lettuce, in the diet of patients suffering from diabetes during the determination of their carbohydrate tolerance makes it desirable to know with considerable accuracy the amount of sugar-forming substance which these foods contain. The recorded analyses permit only a doubtful calculation of the desired information, because among other reasons much of the carbohydrate is "fiber" which presumably is not digested or absorbed by man; the availability of the starch may be more or less uncertain depending upon the extent to which it is liberated from its protecting cell structure by cooking, mastication, and digestion; and because certain organic acids, which by hydrolysis and reduction methods are not determined as carbohydrate, may form sugar after absorption from the intestine. Furthermore, published analyses¹ usually give carbohydrate as determined only by difference, and the combined errors thus fall on this constituent. While such figures represent more or less correctly total carbohydrate, with or without fiber, there is no assurance that they indicate the amount of carbohydrate available to the body when the materials are eaten. Thus, Lusk² fed cauliflower to a phloridzinized dog without detecting sugar production, yet by analysis cauliflower contains 3.7 per cent carbohydrate.

¹ Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Bull.* 28, revised, 1906.

² Lusk, G., *Am. J. Physiol.*, 1910-11, xxvii, 467.

46 Carbohydrate in Certain Vegetables

It was, therefore, decided to determine by more direct methods the sugar-forming material in those vegetables which are commonly used in the dietaries of diabetics in order to be able to calculate more exactly the available carbohydrate in such diets.

Four methods have been used for the determination of carbohydrate in foodstuffs. First is the method most commonly used by which carbohydrate is calculated by difference after direct determination of fat, protein ($N \times 6.25$), ash, water, and insoluble fiber; second, direct hydrolysis by mineral acid and the subsequent determination of reducing sugar; third, preliminary hydrolysis of starch by diastase to dextrins, maltose, and glucose, followed by copper reduction and determination by polariscope or by acid hydrolysis for the conversion to glucose which is then determined by reduction; and fourth, analysis by means of the phloridzinized animal. We have used the last two methods named.

The use of the phloridzinized dogs should give results indicating the total available sugar-forming substance, including sugar formed by such substances as protein and organic acids; while the results of the diastase method should indicate only reducing sugars, preformed, and from the hydrolysis of starch.

Analysis in Vitro by Diastase and Copper Reduction.

The direct hydrolysis of the vegetable material by boiling hydrochloric acid is objectionable because of the hydrolysis of the indigestible fiber and because as shown by Davis and Daish³ 5 or 6 per cent of the maltose may be decomposed and lost.

The milder and more selective action of a diastase as the hydrolytic agent for the starch seems therefore preferable, and has been used for our analyses. Of the commercial diastases available, that of the *Aspergillus oryzae*, the "taka-diastase," proved the most satisfactory, in that hydrolysis was more complete. Results with a 10 per cent extract of malt as recommended in the official methods⁴ gave only 90 to 95 per cent with pure starch. Davis and Daish³ used taka-diastase successfully in their determinations of starch in certain leaves.

³ Davis, W. A., and Daish, A. J., *J. Agric. Sc.*, 1914, vi, 152.

⁴ *J. Assn. Offic. Agric. Chem.*, 1916, ii, pt. 1, 110.

For the determination of the sugar formed by the action of diastase, Davis and Daish used the combined methods of reduction and optical activity of the solutions. We have preferred to employ a secondary hydrolysis of the maltose and any residual polysaccharide by heating with a minimum concentration of acid and to determine the resulting glucose by copper reduction. While this acid hydrolysis probably leads to some decomposition of sugar, the loss under the conditions used is not greater than about 2 per cent and is probably within the limit of error of other stages in the procedure. The acid hydrolysis was carried out on the individual portions taken for analysis in centrifuge tubes placed in boiling water bath under which condition there is less destruction of sugar than occurs in boiling with a reflux condenser.

The principles in the determination were: The thorough trituration and maceration of vegetable samples; the action of 0.1 gm. of taka-diastrase on watery samples at 37°C. for 18 hours; filtration, hydrolysis, and the determination of glucose by Fehling's solution, using Bertrand's method for titration of cuprous oxide.

Procedure.

Samples of the vegetables were carefully selected, put through the finest food chopper, and 20 to 50 gm. weighed out, care being taken to retain the water squeezed out in grinding. These samples were put into a flask with reflux condenser and boiled on a hot plate 1 or 2 hours in 100 to 150 cc. of water. After cooking the sediment was macerated to a paste in a mortar. The only vegetable which offered any difficulty was lettuce and it was found impossible to macerate the leaves, even after prolonged boiling. After combining mother liquor and paste, each sample was washed into a 250 cc. flask and boiled again in a water bath 15 minutes, then cooled to 37° C., and 0.1 gm. of taka-diastrase was added to each flask. All samples were incubated 17 hours at 37° C. with a few drops of toluene as a preservative. After incubation the flasks were made up to volume, well shaken, and 100 cc. taken. 5 cc. of a molecular solution of neutral lead acetate were added to precipitate tannins and gums. Samples were filtered, the filtrate being tested with lead for completeness of precipitation. 10 cc. of filtrate—equaling 9.52 cc. of the original—were taken for determination.

The hydrolysis of sugars, reduction of copper, and the titration by Bertrand's method were done in 50 cc. centrifuge tubes.⁵ To 10 cc. of the

⁵ Schaffer, P. A., *J. Biol. Chem.*, 1914, xix, 285.

TABLE I.

Analysis of Starch by Taka-Diastase and Hydrolysis.

Two samples of Kahlbaum's arrowroot starch, dry weight 0.88 gm., were washed into 250 cc. volumetric flasks with 150 cc. of water. Gelatinized 15 minutes in boiling water bath; cooled to 37°C. and 0.1 gm. of commercial taka-diastase added. Samples incubated at 37° for 20 hours.

100 cc. of starch-diastase mixture were taken to which were added 5 cc. of a molecular solution of neutral lead acetate.

10 cc. of filtrate, representing 9.52 cc. of the original, were taken for determination (36.85 mg. of dextrose).

Samples hydrolyzed 2½ hrs., concentration of acid 0.7 N.

0.04 N permanganate.	Copper.	Copper corrected for blanks.	Glucose.	Theoretical.
cc.	mg.	mg.	mg.	per cent
31.3	79.3	73.2	36.9	100.1
31.2	79.0	72.9	36.8	99.8
31.0	78.5	72.4	36.5	99.0
31.4	79.4	73.3	37.0	100.4

TABLE II.

Summary of Analyses by Taka-Diastase.

Vegetable.	Weight of the sample.	No. of samples.	Average glucose in aliquot (9.52 cc.) samples.	Average glucose in samples.	Carbohy- drate as glucose.
	gm.		mg.	gm.	per cent
Potato.....	5	6	35.5	0.930	18.6
Lettuce.....	50	4	18.9	0.495	1.0
Cabbage.....	20	4	33.5	0.877	4.38
Cauliflower.....	30	6	32.1	0.840	2.80
Cabbage, thrice boiled.....	30	5	4.7	0.123	0.4
Cauliflower, " ".....	30	6	10.2	0.267	0.8

TABLE III.

Analyses of Vegetables by Taka-Diastase and by Atwater and Bryant Compared.

	Taka- diastase.	Atwater and Bryant.
Cabbage.....	4.4	4.5
" thrice cooked.....	0.4	
Cauliflower.....	2.8	3.7
" thrice cooked.....	0.8	
Lettuce.....	1.0	2.2

filtrate were added 2 cc. of 7 N hydrochloric acid and water to give a volume of 20 cc. The final concentration of acid was thus 0.7 N. The tubes were placed in a gently boiling water bath 2½ hours. After hydrolysis the acid was almost neutralized with a measured amount of strong sodium hydroxide, 20 cc. of mixed Fehling's solution were added, and the tubes heated in a boiling water bath exactly 10 minutes. Tubes were centrifuged 3 minutes, the excess Fehling's solution was decanted, and cuprous oxide washed twice with 20 cc. of water, centrifuging and decanting each time. After decanting following the last washing, copper was dissolved in 1 to 2 cc. of strong ferric sulfate-sulfuric acid and titrated with 0.04 N potassium permanganate solution. Blanks were always run on 0.1 gm. of taka-diastase and on the mixed Fehling's solution, the titration of blanks being deducted from titration of cuprous oxide of the sample. The number of cc. of 0.04 N permanganate used multiplied by 2.54 gives the mg. of glucose present in 9.52 cc. of sample. The Bertrand titration was checked by titrating solutions of pure anhydrous glucose.

The use of centrifuge tubes simplifies the process considerably but limits the amount of mixed Fehling's solution that can be used. Experiments showed that 20 cc. of mixed Fehling's solution gives accurate results when between 30 and 40 mg. of dextrose are present. So in the determinations the amount of the sample was so arranged as to give amounts of dextrose within these limits as near as possible.

This method applied to pure, dry, arrowroot starch gave results shown in Table I. As applied to potato, cabbage, cauliflower, and lettuce the method gave results shown in Table II.

The results of analyses of cabbage, cauliflower, and lettuce by the use of taka-diastase were lower than the accepted analyses of Atwater and Bryant; this was especially true of lettuce. Table III is a comparison of analyses.

The extensive use of thrice cooked cabbage and cauliflower in the treatment of diabetes suggested the analyses of these as prepared in the diet kitchen according to Joslin.⁶ The results show that 60 to 90 per cent of the carbohydrate is lost in the cooking water.

Experiments with Phloridzinized Animals.

The phloridzinization of dogs was conducted after the method of Sansum and Woodyatt;⁷ namely, 48 hours of complete fasting followed by 1 gm. of phloridzin in oil subcutaneously every 12

⁶ Joslin, E. P., *Treatment of diabetes mellitus*, Philadelphia and New York, 1916.

⁷ Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1916, xxiv, 327.

hours and adrenalin, 0.04 mg. per kilo of body weight of animal, subcutaneously every 6 hours.

Sansum and Woodyatt,⁸ Lusk, Janney,⁹ and others have previously used phloridzinized animals as a method of carbohydrate analysis. The animals were kept in clean metabolism cages and catheterized at the end of each experimental period. The urines were preserved with toluene and made up to 1,000 or 1,500 cc.

Nitrogen in urine was determined by Kjeldahl and sugar by the same technique used with plant extracts, clearing with lead acetate, reduction of Fehling's solution, and titration of copper by Bertrand. All urines were read in a polariscope but more accurate D:N ratios were obtained by determining glucose by reduction.

The urine from some dogs gave low glucose figures when calculated from polariscopic readings. In other animals the difference between the results calculated from polariscope and reduction was not great. In Tables IV and V glucose calculated by both methods is given. In Table IV the difference is not very great, but in Table V it is considerable. It will be seen that the D:N ratios calculated from polariscopic values are low, being below 3.00. The presence of levorotatory substances in dog urine is well known and for that reason the determination of glucose by reduction is more accurate. Determinations by polariscope should not be accepted in such work.

Feeding 200 to 500 gm. of bulky vegetables presented some difficulty. The animals refused to eat them of their own volition, but by mixing melted lard with the cooked vegetables and placing the mixture in the animal's mouth, it would always be quickly and often eagerly swallowed. Any liquor left after feeding the bulky material was fed by stomach tube. No vomiting of feedings was experienced. The vegetables were prepared in the same manner used in analysis by diastase, care being taken to boil down water to a minimum. The results are, therefore, for cooked vegetables. If eaten raw the amount of starch digested would doubtless be less.

⁸ Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1916, xxiv, 23.

⁹ Janney, N. W., *J. Biol. Chem.*, 1915, xx, 321.

The extra sugar in all experiments was calculated by the usual method from the average of the starvation D:N ratios of the periods before and after the feeding periods. At the foot of each table are given these ratios. The starvation ratios multiplied by the nitrogen elimination of the feeding periods gives the sugar arising from the protein and this amount subtracted from the sugar excreted during feeding period gives the extra glucose arising from carbohydrate material fed.

The periods where vegetables were fed were made 24 hours in length. It was found that 24 to 30 hours are required for the elimination of all the extra glucose. This slow elimination of extra sugar from such sources of carbohydrate must be taken into account in any experiments where vegetables are fed to phloridzinized animals. In some of the experiments the amount of extra glucose was very small. But the large amount of material, and careful verification of analysis and calculations have convinced us that these amounts of glucose arose from the material fed.

Table IV gives the result from a dog to which was given glucose as a control. The calculation gives a recovery of from 94 to 107 per cent of the sugar fed. It is of interest to note that the sugar fed in Period IV had been racemized by boiling alkaline phosphate solution until the solution was no longer optically active. In the organism, it was, however, converted again quantitatively into dextroglucose. The results indicate satisfactory recovery of available carbohydrate.

Sansum and Woodyatt⁸ obtained from 50 to 75 per cent of dextrose fed by mouth to phloridzinized dogs; Csonka¹⁰ recovered 100 per cent in his experiments.

Table V shows recovery of 17.3 dextrose from 500 gm. of cauliflower or 3.4 per cent of weight. Although Lusk² found no extra sugar after 20 gm. of cauliflower fed to a phloridzinized animal, the small amount of ingested material may well account for his result. By analysis 2.8 per cent of weight of cauliflower was found to be carbohydrate.

¹⁰ Csonka, F. A., *J. Biol. Chem.*, 1916, xxvi, 93.

TABLE IV.

Dog 7 Phloridzinized after 2 Days of Fasting; Weight, 13 Kilos.

10 gm. of sugar given in Period IV were boiled 3 hours in a di-basic phosphate solution of pH 8. The rotation of this solution by polariscope was zero.

The sugar given in Period VII was 10 gm. of pure anhydrous dextrose dissolved in distilled water. Sugar solutions were given by stomach tube.

Period.	Time.	Total N.	Glucose by polariscope.	D:N	Glucose by reduction.	D:N	Extra sugar.	Remarks.
	1917	gm.	gm.		gm.		gm.	
I	Mar. 6, 6 p.m.-12 m., 6 hrs.	4.58	13.3	2.90	15.0	3.28		
II	Mar. 7, 12 m.-6 a.m., 6 hrs.	4.01	11.0	2.75	12.7	3.17		
III	Mar. 7, 6 a.m.-12 n., 6 hrs.	3.84	11.6	3.02	12.2	3.17		
IV	Mar. 7, 12 n.-6 p.m., 6 hrs.	3.60	19.4	5.18	20.5	5.48	10.7	10 gm. of sugar phosphate mixture given at beginning of Period IV. Extra sugar in parenthesis calculated from polariscope reading.
V	Mar. 7, 6 p.m.-12 m., 6 hrs.	3.44	12.3	3.57	12.8	3.72	(10.4)	
VI	Mar. 8, 12 m.-6 a.m., 6 hrs.	3.71	11.2	3.02	12.1	3.26		
VII	Mar. 8, 6 a.m.-12 n., 6 hrs.	3.65	19.3	5.27	20.2	5.52	9.4	10 gm. of sugar given at beginning of Period VII.
VIII	Mar. 8, 12 n.-6 p.m., 6 hrs.	3.21	10.0	3.11	11.1	3.45	(8.4)	
IX	Mar. 8, 6 p.m.-12 m., 6 hrs.	3.32	10.2	3.07	11.2	3.37		
X	Mar. 9, 12 m.-6 a.m., 6 hrs.	3.48	10.0	2.87	10.8	3.09		

Average D: N of Periods II, III, and VI 3.21.

" D: N " " VI, IX, " X 3.20.

TABLE V.

*Cauliflower.**Dog 5 Phloridzinized after Fasting; Weight, 14.5 Kilos.*

Period	Time.	Total N.	Glucose by polariscope.	D: N	Glucose by reduction.	D: N	Extra sugar.	Remarks.
	1917	gm.	gm.		gm.		gm.	
I	Feb. 13, 6 p.m.- 12 m., 6 hrs.	5.13	21.1	4.11	25.4	4.95		
II	Feb. 14, 12 m.- 6 a.m., 6 hrs.	7.06	20.1	2.85	22.8	3.23		
III	Feb. 14, 6 a.m.- 12 n., 6 hrs.	5.23	13.9	2.66	16.4	3.13		
IV	Feb. 14, 12 n.- 12 n., Feb. 15, 24 hrs.	17.54	64.0	3.65	73.5	4.18	17.3	During first 6 hrs. of Period IV 500 gm. of cauliflower fed. Extra sugar in parenthesis calculated from polariscope readings.
V	Feb. 15, 12 n.- 6 p.m., 6 hrs.	4.74	14.8	3.12	16.8	3.55	(15.1)	
VI	Feb. 15, 6 p.m.- 12 m., 6 hrs.	4.21	12.7	3.02	14.2	3.37		
VII	Feb. 16, 12 m.- 6 a.m., 6 hrs.	4.02	11.7	2.91	13.3	3.31		

Average D: N of Periods II, III, VI, and VII 3.21.

Available carbohydrate 3.6 per cent.

Table VI shows that from 200 gm. of cabbage 10 gm. of glucose were recovered, or 5 per cent of the weight of material fed. By diastase analysis 4.4 per cent was found. The difference is possibly within the limits of the variation in samples, or may indicate the formation of glucose from organic acids or other substances. In the case of both cabbage and cauliflower some-

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what lower results were obtained from analysis than by dog method.

TABLE VI.

Cabbage.

Dog 4 Phloridzinized after Fasting; Weight, 8 Kilos.

Period.	Time.	Total N.	Glucose by re- duction.	D: N	Extra sugar.	Remarks.
	1917	gm.	gm.		gm.	
I	Jan. 31, 12 n.-6 p.m., 6 hrs.	4.27	13.7	3.20		
II	Jan. 31, 6 p.m.-12 m., 6 hrs.	4.01	13.0	3.22		
III	Feb. 1, 12 m.-6 a.m., 6 hrs.	3.71	14.0	3.77		
IV	Feb. 1, 6 a.m.-6 a.m., Feb. 2, 24 hrs.	12.84	57.6	4.48		
V	Feb. 2, 6 a.m.-12 n., 6 hrs.	2.72	10.0	3.68	10.1	100 gm. of cabbage given at beginning of Period IV; 100 gm. 6 hrs. later. A little lard.
VI	Feb. 2, 12 n.-6 p.m., 6 hrs.	1.75	6.3	3.60		Animal exhausted.

Average D: N of Periods III and VI 3.68.

Available carbohydrate 5 per cent.

Tables VII and VIII show that nearly all the carbohydrates may be washed out of the vegetables by cooking. Snyder and coworkers¹¹ cooked whole heads of cabbage, determining the carbohydrate before and after cooking by the Sachasse method, and found a loss of 34 per cent of carbohydrate and fat. The

¹¹ Snyder, H., Frisby, A. J., and Bryant, A. P., *U. S. Dept. Agric., Bull.* 43, 1897.

TABLE VII.
*Thrice Boiled Cabbage.**
Dog 2 Phloridzinized after Fasting; Weight, 12.8 Kilos.

Period.	Time.	Total N.	Glucose by re- duction.	D:N	Extra sugar.	Remarks.
	1917	gm.	gm.		gm.	
I	Feb. 13, 6 p.m.-12 m., 6 hrs.	3.26	14.8	4.54		
II	Feb. 14, 12 m.-6 a.m., 6 hrs.	3.92	16.0	4.08		
III	Feb. 14, 6 a.m.-12 n., 6 hrs.	3.64	14.0	3.84		
IV	Feb. 14, 12 n.-12 n., Feb. 15, 24 hrs.	13.43	50.0	3.72	1.7	During first 6 hrs. of Period IV 300 gm. of thrice boiled cabbage plus a little lard.
V	Feb. 15, 12 n.-6 p.m., 6 hrs.	3.30	13.5	4.08		
VI	Feb. 15, 6 p.m.-12 m., 6 hrs.	3.19	11.8	3.70		
VII	Feb. 16, 12 m.-6 a.m., 6 hrs.	3.11	11.1	3.57		

* Thrice boiled by method of Joslin.*

Average D: N for Periods III, VI, and VII 3.70.

Available carbohydrate 0.5 per cent.

greater losses in our analysis are due to the fine division of the sample before cooking.

Table IX shows the recovery of 5.9 gm. of glucose, or 1.2 per cent of weight, from 500 gm. of spinach. It is regretted that we have no analysis of spinach, but the accepted analysis by difference gives 2.3 per cent carbohydrate. The analysis of lettuce by taka-diastase gave 1.0 per cent carbohydrate. The per cent

as analyzed by difference is 2.2. This data although incomplete would indicate less of available carbohydrate in the green leafy vegetables than earlier analyses indicate.

TABLE VIII.

*Thrice Boiled Cauliflower.**
Dog 6 Phloridzinized after Fasting; Weight, 17 Kilos.

Period.	Time.	Total N.	Glucose by re- duction.	D: N	Extra sugar.	Remarks.
	1917	gm.	gm.		gm.	
I	Feb. 13, 6 p.m.-12 m., 6 hrs.	2.36	17.3	7.3		
II	Feb. 14, 12 m.-6 a.m., 6 hrs.	3.37	13.6	4.0		
III	Feb. 14, 6 a.m.-12 n., 6 hrs.	3.79	13.5	3.55		
IV	Feb. 14, 12 n.-12 n., Feb. 15, 24 hrs.	16.62	59.7	3.59	4.3	500 gm. of thrice boiled cauliflower given dur- ing first 3 hours of this period. A little lard added.
V	Feb. 15, 12 n.-6 p.m., 6 hrs.	3.78	12.3	3.25		
VI	Feb. 15, 6 p.m.-12 m., 6 hrs.	3.68	12.4	3.37		
VII	Feb. 16, 12 m.-6 a.m., 6 hrs.	3.46	11.0	3.18		

* Thrice boiled by method of Joslin.*

Average D: N of Periods III, V, VI, and VII 3.34.

Available carbohydrate 0.8 per cent.

TABLE VII.
*Thrice Boiled Cabbage.**
Dog 2 Phloridzinized after Fasting; Weight, 12.8 Kilos.

Period.	Time.	Total N.	Glucose by re- duction.	D:N	Extra sugar.	Remarks.
	1917	gm.	gm.		gm.	
I	Feb. 13, 6 p.m.-12 m., 6 hrs.	3.26	14.8	4.54		
II	Feb. 14, 12 m.-6 a.m., 6 hrs.	3.92	16.0	4.08		
III	Feb. 14, 6 a.m.-12 n., 6 hrs.	3.64	14.0	3.84		
IV	Feb. 14, 12 n.-12 n., Feb. 15, 24 hrs.	13.43	50.0	3.72	1.7	During first 6 hrs. of Period IV 300 gm. of thrice boiled cabbage plus a little lard.
V	Feb. 15, 12 n.-6 p.m., 6 hrs.	3.30	13.5	4.08		
VI	Feb. 15, 6 p.m.-12 m., 6 hrs.	3.19	11.8	3.70		
VII	Feb. 16, 12 m.-6 a.m., 6 hrs.	3.11	11.1	3.57		

* Thrice boiled by method of Joslin.*

Average D:N for Periods III, VI, and VII 3.70.

Available carbohydrate 0.5 per cent.

greater losses in our analysis are due to the fine division of the sample before cooking.

Table IX shows the recovery of 5.9 gm. of glucose, or 1.2 per cent of weight, from 500 gm. of spinach. It is regretted that we have no analysis of spinach, but the accepted analysis by difference gives 2.3 per cent carbohydrate. The analysis of lettuce by taka-diastrase gave 1.0 per cent carbohydrate. The per cent

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as analyzed by difference is 2.2. This data although incomplete would indicate less of available carbohydrate in the green leafy vegetables than earlier analyses indicate.

TABLE VIII.

*Thrice Boiled Cauliflower.**
Dog 6 Phloridzinized after Fasting; Weight, 17 Kilos.

Period.	Time.	Total N.	Glucose by re- duction.	D: N	Extra sugar.	Remarks.
	1917	gm.	gm.		gm.	
I	Feb. 13, 6 p.m.-12 m., 6 hrs.	2.36	17.3	7.3		
II	Feb. 14, 12 m.-6 a.m., 6 hrs.	3.37	13.6	4.0		
III	Feb. 14, 6 a.m.-12 n., 6 hrs.	3.79	13.5	3.55		
IV	Feb. 14, 12 n.-12 n., Feb. 15, 24 hrs.	16.62	59.7	3.59	4.3	500 gm. of thrice boiled cauliflower given dur- ing first 3 hours of this period. A little lard added.
V	Feb. 15, 12 n.-6 p.m., 6 hrs.	3.78	12.3	3.25		
VI	Feb. 15, 6 p.m.-12 m., 6 hrs.	3.68	12.4	3.37		
VII	Feb. 16, 12 m.-6 a.m., 6 hrs.	3.46	11.0	3.18		

* Thrice boiled by method of Joslin.*

Average D: N of Periods III, V, VI, and VII 3.34.

Available carbohydrate 0.8 per cent.

TABLE IX.
Spinach.
Dog 1 Phloridzinized after Fasting; Weight, 17 Kilos.

Period.	Time.	Total N.	Glucose	D: N	Extra sugar.	Remarks.
	1917	gm.	gm.		gm.	
I	Feb. 6, 6 p.m.-12 m., 6 hrs.	3.96	14.8	3.74		
II	Feb. 7, 12 m.-6 a.m., 6 hrs.	4.21	14.0	3.32		
III	Feb. 7, 6 a.m.-12 n., 6 hrs.	4.11	13.5	3.28		
IV	Feb. 7, 12 n.-12 n., Feb. 8, 24 hrs.	16.28	57.0	3.53	5.9	100 gm. of spinach plus a little lard, given dur- ing the first 6 hours.
V	Feb. 8, 12 n.-6 p.m., 6 hrs.	3.78	14.5	3.83		
VI	Feb. 8, 6 p.m.-12 m., 6 hrs.	3.67	12.4	3.36		40 gm. of lard given at beginning of this pe- riod.
VII	Feb. 9, 12 m.-6 a.m., 6 hrs.	3.17	10.2	3.21		

Average D: N of Periods II, III, VI, and VII 3.29.

Available carbohydrate 1.2 per cent. •

SUMMARY.

Vegetables usually used in low carbohydrate diets for diabetic patients were analyzed by the use of diastase and copper reduction, and by feeding to phloridzinized dogs with the following results.

of results which at first appeared inconsistent. In a series of about twenty experiments, only about half resulted in an increase in blood sugar and also in plasma sugar. The amount of hemorrhage was of about the same order of magnitude, or so nearly so that no consistent relationship appeared evident between body weight, amount of blood loss, and production of increase in sugar. In other words, the occurrence of hyperglycemia did not appear to be dependent solely upon overreaching a given volume of blood loss per body weight, but upon some controlling factor being more affected in some instances than in others. Thus it might be that the quantity of blood loss was near the limiting threshold of tolerance, such that only in a portion of the whole series of animals did there occur hyperglycemia. The rest of the series might logically be expected to have developed hyperglycemia with a larger blood loss.

Having in mind the facts recently emphasized by Murlin and Sweet (6) and by McDanell and Underhill (7) that an increase in acids was productive of a greater susceptibility toward the production of hyperglycemia and that injections of alkalis retard or prevent the development of hyperglycemia by ether, epinephrine, and other agents, it was thought that a change in the acid-base equilibrium in the body, indicated by that in the blood, may be the immediate determining factor.

The work of this paper is the report of an effort to gain further insight into some of the factors involved in the control of sugar mobilization after hemorrhage with contributory evidence regarding a chemical controlling mechanism of sugar balance.

Methods.

Blood and Plasma Sugar.

Rabbits alone were used in this series of experiments. Most of the previous study of the effects of hemorrhage on blood sugar by earlier investigators have been made on rabbits. They do not as a rule give much or any evidence of excitement on handling, are easily bled from the ear vein without any anesthetic and without struggling, and are well supplied with hepatic glycogen. Blood was drawn from the ear by cutting into but not through the marginal ear vein. The blood was collected in small wide-mouthed bottles or centrifuge tubes containing enough potassium oxalate to prevent clotting. The subsequent hemorrhages for the purpose of

studying the effects of the loss of blood were made after a period of 1 hour, more or less, as specified in the tables.

Anesthetics were purposely avoided even at the expense of increased difficulty of withdrawing of blood. The use of an anesthetic adds complications since it is impossible to standardize anesthesia and its effects on the blood contents and on activity of body cells. Different animals respond somewhat differently to ether even though care is especially taken in regard to intensity of anesthesia and its duration. This has been often observed in study of hyperglycemia following ether anesthesia. While hyperglycemia always follows, its grade varies considerably in comparing different animals. Consequently the additional variable incurred by anesthetics was purposely avoided.

For blood sugar determinations, the method of Lewis and Benedict (8), as modified by Myers and Bailey (9), was used. The Shreiner colorimeter was used for color comparison. This instrument was found to be very satisfactory for this purpose especially after placing tubes of black paper in the inner tubes to prevent the halo of paler color coming in through the sides.

Recent investigations (10) have shown some difficulty in the use of the method of Benedict for absolute quantitative estimations of reducing substances since the amount of color produced does not appear to be strictly proportional to sugar present, requiring corrections for wide limits. Yet on the use of the method for relative rather than absolute values the method appears to be entirely adequate and possesses the great advantage of simplicity and quickness when comparative values are to be utilized.

For plasma sugar determination, the blood was centrifuged as quickly as possible after hemorrhage to avoid glycolysis. Proportions of cells to plasma were determined by use of graduated centrifuge tubes, stoppered and centrifuged a fixed length of time, or until a constant volume of cells was obtained. This reading, while not so accurate perhaps as the hematocrit or specific gravity measurements, was deemed sufficiently accurate for the purposes at hand.

In the consideration of blood sugar it is recognized that blood contains reducing substances other than sugar, but fermentation tests have demonstrated that most of the reducing substance is sugar, and while other substances may be appreciable, perhaps of more importance than has been generally recognized, the inexactness in names of substances measured does not at all invalidate the fundamental arguments of this paper.

Alkaline Reserve Capacity.

The alkali-acid status of the blood was assayed by determinations of the alkaline reserve capacity of whole blood rather than CO_2 content of whole blood, or of plasma, or the CO_2 -binding capacity of plasma alone. It is to be realized that venous blood is *more* or *less* venous according to the local conditions at the moment, and that therefore venosity of venous blood is itself a variable. This would appear also a considerable item

when blood is drawn for clinical purposes from the basilic vein after the application of a constrictor to distend the vein. Consequently it would seem desirable to reduce the condition of drawn blood to a standard. This was on tentative theoretical grounds approximated by rendering venous blood arterial by allowing it to come to a state of equilibrium with alveolar air which essentially renders the venous blood arterial and furthermore to a standard degree of arterialization.

The alkaline reserve CO_2 apparatus of Van Slyke (11) was used, though the technique has been somewhat adapted. Instead of using 5 per cent sulfuric acid, a 20 per cent solution of tartaric acid was used as recommended by Henderson and Morriss (12) for their special method. This acid does not seriously precipitate proteins as does sulfuric acid and consequently the extractor is very easily cleaned after each extraction by first running in 2 or 3 cc. of 10 per cent sodium hydroxide solution for the absorption of the carbon dioxide extracted from the acid-blood mixture. Then by the use of boiled distilled water the whole apparatus is readily cleaned by two or three washings. Any emulsified mercury is run out of the apparatus to be washed and dried by cotton and filter paper.

Again, owing to the fact as explained by Van Slyke that carbon dioxide is not completely extracted by fifteen successive shakings but should be corrected for unextracted carbon dioxide or by reextraction, I have adopted the procedure of allowing the mercury and acid-blood mixture to fall to the middle or lower third of the lower side reservoir after thorough mixing in the larger upper reservoir as Van Slyke directed, then raising the side reservoir to allow the fluid to reach just the upper aperture of the lower gas-cock. This now is turned allowing mercury to rise to bring the extracted gas to atmospheric pressure. Reading is now made, then the process is repeated twice after which the readings are usually constant for any given specimen.

In this paper corrections as recommended by Van Slyke are not given, since it is not absolute amounts of gas at standard conditions of saturation (temperature and atmospheric pressure) but it is *variations* in these amounts that under the same conditions indicate sufficiently for our purpose physiological variations. Furthermore normal differences in reserve capacity of different animals appear to be between considerable limits, especially in

herbivorous animals like the rabbit, while smaller but real variations are observed in the dog and in man. This relative measure obviously would not be satisfactory in a precise determination of critical points such as the lower limiting threshold of capacity from which an animal may or may not recover.

Effect of Hemorrhage on Hyperglycemia Production.

In performing a series of hemorrhages in rabbits to determine the constancy of production of hyperglycemia after a blood loss of a fairly constant proportion of total blood volume, there

TABLE I.
Blood Sugar before and after Hemorrhage. Control Experiments.

Rabbit No	Weight.	Sex.	Blood sugar per cc.				Cells in whole blood.		Hemor- rhage per kilo.	Total hemor- rhage.
			Whole blood.		Plasma.		Before.	After.		
			Before.	After.	Before.	After.				
	kg.		mg.	mg.	mg.	mg.	per cent	per cent	cc.	cc.
27	1.6		1.18	1.02	1.59	1.26	31.0	27.0	18.7	30
28	1.4		1.17	1.26	1.51	1.68	34.0	23.0	21.4	30
29	1.2		1.23	1.59	1.51	1.68	31.6	21.3	25.0	30
31	1.43	♂	1.06	1.00	1.45	1.27	43.0	35.0	15.4	22
32	1.98	♀	1.22	1.29	1.38	1.51	28.0	27.0	10.1	20
33	1.1	♀	1.07	1.46	1.27	1.46	36.0	29.0	18.1	20
34	1.5		1.33	1.37	1.46	1.37	27.0	21.0	16.6	25
35	1.9	♀	1.29	1.29	1.51	1.37	37.0	31.5	13.1	25
36	1.3	♂	1.13	1.41	1.26	1.41	35.0	29.0	11.5	15
37	1.2		1.04	1.03	1.17	1.03	34.0	28.0	16.6	20
38	1.2	♀	1.37	1.61	1.61	1.80	37.5	27.0	16.6	20
40	1.48	♀	1.22	1.22	1.96	1.33	37.5	30.0	12.1	18
41	1.5	♂	1.29	1.46	1.35	1.56	30.7	28.7	13.3	20
42	1.7	♀	1.14	1.14	1.13	1.15	32.5	27.5	12.9	22
43	3.0	♀	1.08	1.02	1.17	1.14	38.0	34.5	7.0	22

appeared great irregularity of its production. As seen in Table I, approximately half the experiments developed a rise in blood sugar concentration of a significant amount while the remainder of the series suffered no demonstrable change in sugar content. This fact leads to the conclusion that it is not the mere loss of a given volume of blood *per se* but upon some other factor affected

TABLE II.
Blood Sugar and Alkaline Reserve Capacity of Whole Blood in Hemorrhage.

Animal No.	Sex.	Weight.	Time.		Hemorrhage.*	Blood sugar per cc. of blood.	Reserve capacity.	Notes.
		kg.	1919		cc.	mg.		
114B40	♀	2.4	Nov. 3,	2.00 p.m.	5.0	1.08	0.59	
				3.00 "	5.0	1.08	0.63	
				4.00 "	30.0	1.37	0.62	
				4.45 "	5.0	1.29	0.56	
115B40	♀	1.9	" 4,	2.00 "	5.0	1.22	0.67	
				2.45 "	5.0	1.26	0.70	
				3.30 "	26.0	1.67	0.66	
				4.15 "	5.0	2.38	0.63	
116B40	♂	3.1	" 6,	2.20 "	5.0	1.13	0.66	
				3.00 "	5.0	1.37	0.66	
				3.45 "	30.0	1.46	0.66	
				4.30 "	5.0	1.67	0.66	
				5.15 "	30.0	1.41	0.66	
				5.45 "	5.0	1.37	0.64	
117B40	♀	2.7	" 7,	2.00 "	5.0	1.73	0.63	
				2.50 "	5.0	1.61	0.65	
				3.45 "	30.0	1.56	0.66	
				4.30 "	5.0	1.73	0.65	
				5.15 "	30.0	2.16	0.60	
				5.45 "	5.0	2.82	0.54	
58B40	♀	1.8	Aug. 13,	3.15 "	4.0	1.55	0.77	
				4.00 "	4.0	1.46	0.73	
				5.00 "	18.0	1.50	0.71	
				6.00 "	4.0	1.67	0.55	
			" 14,	3.00 "	4.0	1.37	0.68	
				" 15,	9.30 a.m.	4.0	1.46	0.65
			11.00 "		18.5	1.41	0.64	
			2.00 p.m.	4.0	1.74	0.68		
			3.00 "	14.0	2.05	0.62		
			4.30 "	4.0	1.81	0.64		
5.30 "	14.0	3.01	0.42					

* In Tables II, IV, and VI when the hemorrhage exceeded 5 cc. in any instance the last 5 cc. drawn were used for analysis.

TABLE II—*Concluded.*

Animal No.	Sex.	Weight. kg.	Time.	Hemorrhage.	Blood sugar per cc. of blood.		Reserve capacity.	Notes.
				cc.	mg.			
88B40	♀	1.5	1919					Fasting 24 hrs. before and throughout the experiment.
			Aug. 24,	4.00 p.m.	4.0	1.16	0.63	
				5.00 "	29.0	1.41	0.60	
				6.00 "	4.0	1.67	0.57	
				7.00 "	11.0	2.38	0.52	
64B40	♀	1.7	"	25, 11.00 "	4.0	1.56	0.66	
			"	21, 2.30 "	4.0	1.33	0.56	
				5.30 "	27.0	2.05	0.44	
			"	22, 2.30 "	4.0	1.29	0.72	
				3.30 "	4.0	1.37	0.68	
				4.30 "	16.0	1.67	0.60	
				6.00 "	14.0	3.01	0.46	
			"	23, 9.00 a.m.	5.0	1.37	0.63	
61B40	♀	1.4	Aug. 18,	2.00 p.m.	4.0	1.19	0.63	
				3.00 "	4.0	1.29	0.61	
				4.00 "	25.0	1.41	0.64	
				5.00 "	4.0	1.88	0.63	
				6.00 "	19.0	2.51	0.50	
			"	19, 2.15 "	4.0	1.14	0.68	

in some instances by the loss of blood. Extending the hemorrhage to a sufficient amount invariably gives rise to a marked hyperglycemia (Table II). A surprising feature, in a number of the experiments, is the early appearance of hyperglycemia, many times being quite marked at the termination of a hemorrhage as determined by the analysis of specimens of blood obtained at the termination of the hemorrhage lasting from 10 to 15 minutes. This has also been observed by Andersson (3). In this connection it may be stated that such early appearances of hyperglycemia did not appear to be dependent upon emotional states, at least as far as these are manifested by outward signs.

Because of the view of Murlin and Underhill that the acid-base balance in the blood, and in turn in the body cells, particularly in the liver, determines the mobilization of dextrose from its storage reservoirs of glycogen, it was anticipated that a hemorrhage sufficient to produce hyperglycemia might produce changes in the acid-base balance of demonstrable grade. Consequently, studies were made simultaneously of blood sugar and of alkaline reserve capacity of whole blood. From Table II it is seen that this is the case. Those experiments in which a rise in sugar appeared were usually those in which there occurred a fall in alkaline reserve capacity of whole blood. The fall in reserve capacity, furthermore, usually appeared as soon as there appeared a rise in sugar. Those instances in which no rise of sugar appeared usually did not present a fall in reserve capacity.

Furthermore, after 12 to 24 hours, whether the animals had or had not been kept from food before and during the experiment, the sugar content fell to normal while the reserve capacity rose to and sometimes exceeded normal for that animal. The two values appeared to bear a reciprocal relationship. Fasting to a moderate extent (with access to water) from 24 to 48 hours did not prevent a return to normal values for both sugar and reserve capacity. This is taken to mean that a compensation had taken place within the body cells perhaps by hepatic action in ammonia formation from protein metabolism. This could liberate an equivalent amount of sodium bicarbonate and restore the neutrality balance.

Although no quantitative study was made of the respiratory volume, I could in no case foretell the onset or occurrence of hyperglycemia by excitement or struggling, or hyperpnea in certain of the animals in which this occurred. In other words, hyperpnea or struggling, such as occurred in our tame, frequently handled rabbits, was not necessarily concomitant with either a demonstrable fall in reserve or rise in sugar. The conclusion is not made, however, that sufficiently prolonged excitement and hyperpnea might not of itself have produced parallel blood changes. Such a problem concerns this thesis only to the extent of not being the causative factor within these series of experiments.

Effect of Alimentary Administration of Alkalies and Acids.

So far in this investigation there appears a reciprocal relationship between the rise in blood (or plasma) sugar and the fall in alkaline reserve capacity of whole blood after hemorrhage. Which of these two factors is causative and which resultant is not directly answerable from the preceding experiments. This question is approached experimentally from two directions; first, by considering that the fall in reserve capacity of blood reflects the acid-base balance in body cells and hence represents the cause of the hyperglycemia, namely cellular acidosis, second, by considering

TABLE III.

Effect on Blood Sugar of Administration of 0.6 Gm. of NaHCO₃ by Stomach 2 to 3 Hrs. before Hemorrhage.

Rabbit No.	Weight.	Sex.	Blood sugar per cc. of blood.				Cells in whole blood.		Hemorrhage per kilo.	Total hemorrhage.
			Whole blood.		Plasma.		Before.	After.		
			Before.	After.	Before.	After.				
			mg.	mg.	mg.	mg.				
46	1.3	♂	1.19	1.20	1.10	1.16	27.0	21.0	15.3	20
47	1.98	♀	1.26	1.33	1.46	1.41	33.3	28.2	10.1	20
48	1.98	♂	1.33	1.26	1.46	1.40	41.0	35.3	10.1	20
54	1.32	♀	1.26	1.26	1.37	1.39	35.5	30.0	15.1	20

hyperglycemia after hemorrhage to cause the fall in alkaline reserve capacity of whole blood by virtue of incomplete oxidation of metabolites.

On the basis of a fall in reserve by any internal mechanism acting as the causative factor it was thought that by increasing the potential alkali in the body animals should be less subject to hyperglycemia after hemorrhage than normal controls. Hence capsules of sodium bicarbonate were given by mouth and later the animals subjected to hemorrhage of the same order of magnitude as in the preceding series. As seen from Tables III and IV hyperglycemia was, indeed, retarded in appearance. Quite by accident, following overenthusiasm over this means of medica-

tion, it was found that large quantities of bicarbonate actually facilitate the appearance of hyperglycemia on hemorrhage. This, however, appears readily explicable on the basis of Moore's work (13), in which he observes a circulatory injury by excessive tissue alkalosis which in this instance could readily explain the rise in sugar values by hepatic congestion, particularly in conjunction with a reduction in "basal blood flow" from hemorrhage as proposed by Gesell (14).

TABLE IV.

Blood Sugar and Alkaline Reserve Capacity of Whole Blood after Alkali Administration.

Animal No.	Weight.	Sex.	Time.	Hemor- rhage.	Blood sugar per cc. of blood.	Reserve capac- ity.	NaHCO ₃
	kg.		1919	cc.	mg.		gm.
101B40	2.6	♀	Oct. 17, 2.45 p.m.				2.1
			4.45 "	5	1.26	0.78	
			5.30 "	5	1.33	0.85	
			6.00 "	45	1.33	0.77	
109B40	1.9	♀	" 27, 9.10 a.m.				2.1
			10.10 "	5	1.29	0.93	
			11.00 "				0.7
			11.05 "	5	1.45	0.76	
			11.45 "	29	2.51	0.72	
112B40	2.1	♀	" 28, 8.30 "				0.7
			9.10 "	5	1.13	0.69	
			10.20 "	5	1.03		
			10.45 "	30	1.05	0.74	

If the basis just proposed is logical, then a diminution of body reserve in alkali by administration of acids should render the animals more subject to hyperglycemia by hemorrhage. This line of attack requires careful control since no one doubts that acids do produce of themselves hyperglycemia when given in sufficient quantity. Consequently hemorrhage was attempted after preliminary examinations had demonstrated that sugar and reserve values were approaching normal. This eliminates the

danger of development of further hyperglycemia from absorption of acid rather than from hemorrhage itself. Tables V and VI illustrate the effects of hemorrhage after acid (KH_2PO_4) administration by mouth.

While both alkali and acid administration are coupled with danger of complicating factors, it is given as contributory evidence on this thesis.

TABLE V.

Effect on Blood Sugar of Administration of 0.6 Gm. of KH_2PO_4 2 Hrs. previous to First Hemorrhage.

Rabbit No.	Weight.	Sex.	Blood sugar per cc. of blood.				Cells in whole blood.		Hemorrhage per kilo.	Total hemorrhage.
			Whole blood.		Plasma.		Before.	After.		
			Before.	After.	Before.	After.				
			kg.	mg.	mg.	mg.				
49	1.9	♀	1.46	1.61	1.74	1.88	37.0	32.6	10.5	20
50	2.6	♀	1.46	1.61	1.59	1.74	32.3	30.6	7.6	20
51a*	2.6	♀	1.19	1.20	1.37	1.39	41.5	38.0	7.6	20
51b	2.6	♀	1.46	1.50	1.50	1.65	31.0	21.0	7.6	20
53	2.2	♀	1.46	1.56	1.56	1.74	33.5	30.0	9.0	20
84a†	2.8	♀	0.98	0.94					1.0	3
84b	2.8	♀	1.05	1.05					1.0	3

* Large fat rabbit with full stomach. This animal was kept from food for 18 hrs., when the experiment was repeated as No. 51b.

† In order to answer a possible objection to this series, that the increase in blood sugar after hemorrhage may be due to the continued absorption or activity of the acid rather than to the effects of hemorrhage, Rabbit 84a was fed the 0.6 gm. of KH_2PO_4 as in the rest of the series, but differing from these in that a minimum amount of blood was withdrawn. The test was repeated after 24 hrs. fasting, similar to No. 51b. The blood sugar did not rise at either time, eliminating, at least as far as one experiment may be considered as control, the direct effects of acid alone as a possible cause of the increased sugar.

TABLE VI.

Effect on Blood Sugar and Alkaline Reserve Capacity of Whole Blood of Acid Administration.

Animal No.	Weight.	Sex.	Time.	Hemorrhage.	Blood sugar per cc. of blood.		Alkaline reserve capacity.	KH ₂ PO ₄	Notes.
				cc.	mg.				
103B40	2.1	♂	1919 Oct. 20, 9.30 a.m.					0.8	Struggled.
			10.30 "	5	1.03	0.60			
			11.30 "	5	1.08	0.45			
			1.45 p.m.	5	0.98	0.63			
			2.40 "	30	1.25	0.56			
107B40	2.1	♀	" 23, 8.30 a.m.					0.8	"
			10.00 "	5	1.03	0.54			
			10.30 "	5	1.00	0.60			
			10.50 "	28	1.13	0.59			
			11.30 "	5	1.05	0.55			
113B40	2.1	♀	" 28, 3.10 p.m.					0.8	
			4.00 "	5	1.46	0.62			
			4.45 "	5	1.46	0.59			
			5.30 "	29	1.74	0.58			

Effects of Dextrose Injections.

Taking up the problem from the point of view that the hyperglycemia is causative and the fall in reserve capacity is resultant, we performed some experiments by observing the values of blood sugar and alkaline reserve after dextrose injections. As seen from Table VII, the blood sugar was increased but with no detectable change in reserve capacity. It is not, however, inferred that by using prolonged injections and in greater quantities that an acidosis in the newer sense may not be produced (15), yet it does not seem probable from these results that the sugar increase in this investigation is the cause but is rather the result of changes of cellular reaction.

TABLE VII.

Effect on Blood Sugar and Alkaline Reserve Capacity of Whole Blood of Dextrose Injections.

Animal No.	Weight.	Sex.	Time.	Hemorrhage.	Blood sugar per cc. of blood.	Alkaline reserve.	20 per cent dextrose injected intravenously.
	kg.		1919	cc.	mg.		cc.
92B40	2.4	♀	Oct. 7, 10.30 a.m.	5	1.35	0.70	5
			1.30 p.m.	5	1.46	0.65	
			2.30 "	5	1.54	0.65	
			3.00 "				
			3.30 "	5	1.96	0.65	
			4.30 "	5	1.54	0.62	
94B40	2.5	♀	" 8, 1.20 "	5	1.46	0.68	5
			2.20 "	5	1.37	0.64	
			3.00 "				
			3.30 "	5	1.76	0.68	
			5.00 "	5	1.37	0.54	
96B40	2.0	♀	" 9, 2.00 "	5	1.33	0.70	6
			3.00 "	5	1.33	0.67	
			3.40 "				
			4.00 "	5	2.05	0.65	
			5.00 "	5	1.37	0.63	

DISCUSSION.

The results of the experiments described appear to indicate that hemorrhage produces a rise in blood sugar by changes in the state of acid-base balance in body cells, which state is fairly well reflected in corresponding changes in the general circulation. The chief seat of action is probably the liver, for this is the location of glycogen storage most readily affected. Furthermore, the observations of Schenck have shown that ligation of liver vessels prevents hyperglycemia from hemorrhage. The observations of Gesell that hemorrhage lowers alkaline reserve capacity of plasma led him to conclude this was responsible for tissue asphyxia by the diminished "basal blood flow." This tissue asphyxia is effective presumably chiefly in the liver, and hence may be restated by speaking of local changes in acid-base balance in favor of intracellular acidity, a condition generally

thought adequate to facilitate glycogenolysis. A change in hepatic blood might be far more instructively studied since it is conceivable that local changes might be masked to a considerable extent by mixing with systemic blood. Direct study of portal or hepatic artery blood *versus* hepatic vein blood does not at present appear feasible since such an investigation would necessitate extensive abdominal trauma together with anesthesia both of which factors involve serious contraindications towards direct observation.

From the observations (1) that a rise in blood sugar (also plasma sugar) occurs simultaneously with a fall in alkaline reserve capacity of whole blood, (2) that alkalies administered by mouth, which enrich the portal vein blood in alkali, retard the onset of hyperglycemia, while (3) acids by the same route increase the susceptibility to hyperglycemia on hemorrhage, and further (4) that artificial enrichment of blood in sugar by injection in small quantities does not immediately produce a fall in reserve, the conclusion seems warranted that hemorrhage produces hepatic asphyxia in such a way that acids accumulate in liver cells and there promote glycogenolysis. These conditions then lay the responsibility of the rise in sugar upon the fall in alkali in cells. This fall in alkali in cells is fairly well reflected in the state of the blood in the general circulation since all tissues are more or less subjected to the same diminution of basal blood flow, yet local hepatic changes may not necessarily be quantitatively determined by systemic blood study owing to the factor of mixture of hepatic vein blood with systemic blood. The increase in sugar would not appear to be due to concentration of sugar in the blood owing to passage of water from the circulation as emphasized by Epstein (16) in certain conditions since the proportion of plasma to whole blood or to corpuscles may be found much increased $\frac{1}{2}$ to 1 hour after hemorrhage, while both the whole blood and the plasma contain an increased quantity of sugar.

The author believes that the evidences presented support the contentions of Murlin and of Underhill and their collaborators that the acid-base balance in the blood and ultimately that in the body cells, particularly the liver, is one important factor determining the state of glycogenolysis as well as glycogenesis, and that further the hyperglycemia of hemorrhage can be ration-

ally explained not by invoking obscure factors such as shock, depletion, anemia, or emotional states, but on the basis of a disturbance in normal neutrality.

SUMMARY.

1. Whole arterialized blood was used in a study of alkaline reserve capacity.

2. Hemorrhage produces, if its grade is properly chosen, a reciprocal change in both a fall in the alkaline reserve capacity of whole blood and rise in blood and plasma sugar concentration.

3. Hyperglycemia and fall in reserve capacity of whole blood frequently appear within a few minutes after hemorrhage.

4. Recovery from both the reduction in alkaline reserve capacity of whole blood and the hyperglycemia occurs promptly within a relatively few hours even in the limited fasting animal.

5. Hemorrhage produces hyperglycemia more readily when the total bodily alkaline reserve is diminished by administration of acid and less readily when proper amounts of alkali are given.

6. One effective cause of sugar changes in the blood appears to be a disturbance in the acid-base balance in tissue cells indicated by that of the circulating blood in general.

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HEMOGLOBIN.

I. OPTICAL CONSTANTS.

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INTRODUCTION.

In 1873, Vierordt (1) announced the definite relation of the concentration of colored solutions to the extinction coefficients of the same, in the spectrophotometer. He expressed the relation in the formula $A = \frac{c}{a}$ in which A represents the absorption constant for the substance, c , the concentration in gm. per cc., and a , the extinction coefficient. (By later writers, E is used to indicate the extinction coefficient.)

The extinction coefficient is the reciprocal of the thickness of solutions required to reduce light of unit intensity to that of 0.1 unit intensity. This epoch-making work of Vierordt made possible a new line of investigation in connection with substances forming colored solutions. Among various substances studied was hemoglobin. Von Noorden (2), Otto (3), Lambing (4), Hüfner (5), de Saint-Martin (6), Bardachzi (7), Aron and Müller (8), Butterfield (9), and Letsche (10) made a study of hemoglobin and compared the hemoglobin from some of the different species. The results that these investigators obtained did not vary sufficiently to show that the hemoglobins differed markedly in so far as their absorption coefficients were concerned. The work of Korniloff (11), Sczelkow (12), Krüger (13), and Velichi (14) seemed to indicate that the hemoglobins were not all the same since they obtained different extinction coefficients for some of them. Sczelkow explained the differences obtained by him on the basis of differences in concentration of the hemoglobin solutions he employed.

The work of Reichert and Brown (15) gave evidence that crystal forms of the hemoglobins from various species were so different that it was possible to identify species by them. Marshall and Welker (16) discovered an improvement in the method for the preparation of hemoglobin crystals. As prepared by the older methods, the solutions of hemoglobin always contain small amounts of other colloids. These colloids were removed by treatment with aluminium hydroxide cream. This treatment yielded hemoglobin solutions in the case of nearly all species which would crystallize readily. With the difference in crystal form in mind and with a fairly easy method for obtaining crystallized hemoglobin available, the study of the absorption coefficients of the hemoglobin of various species was undertaken.

EXPERIMENTAL.

Blood was drawn into a flask and defibrinated by shaking with glass beads. The defibrinated blood was strained through cheese-cloth and then centrifugalized until the corpuscles were fairly well sedimented. The corpuscles were washed with isotonic sodium chloride solution three or four times, thus removing the major portion of the serum protein. The washed corpuscles were laked by means of ether. This was added, a few drops at a time, and the liquid agitated to insure thorough mixing, and the process continued until a clear solution was obtained. If the solution was so concentrated as to be viscid, it was diluted with water and then treated with an approximately equal volume of aluminium cream. The cream was thoroughly mixed with the solution, and the mixture was then placed on a filter. The solutions of hemoglobin thus prepared were quite clear. They were cooled to a temperature of about $0^{\circ}\text{C}.$, and treated with absolute alcohol which had also been cooled to about $0^{\circ}\text{C}.$ until the percentage of alcohol amounted to from 20 to 30 per cent. The percentage of alcohol required depended upon the concentration of the hemoglobin solution, and also upon the solubility of the particular hemoglobin. Solutions of hemoglobin thus treated with alcohol were allowed to stand at a temperature of a few degrees below $0^{\circ}\text{C}.$ until crystallization was complete. The crystals were then loosened from the

sides of the flask containing the solution, by agitation of the liquid. The crystals were washed by means of 25 per cent alcohol at 0°C. by decantation or by centrifugalization in a cup (17) specially built for this purpose. It is necessary that this low temperature be maintained while the hemoglobin is being washed with the diluted alcohol. If the temperature rises appreciably above 0° the hemoglobin is changed from a crystalline substance over into an amorphous substance. Some of the crystals were roughly dried by placing them on absorbent paper, and transferred to a weighing bottle of known weight. A sample of the moist hemoglobin was weighed out, dissolved in a small quantity of 0.1 per cent sodium carbonate solution, transferred quantitatively to a standard 10 cc. volumetric flask, and diluted to the mark with distilled water. After thorough mixing, a trial dilution of this fluid was made in such a way as to make the total dilution such that, when placed in the spectrophotometer, the readings would be somewhere between 70 and 80, that is, in that portion of the curve where, according to experience, the readings were most accurate. After determining approximately what the dilution should be to bring the reading at that portion of the curve indicated, an accurate dilution was made and this placed in the spectrophotometer and the readings were taken. The figures given for the optical constants represent the mean of ten readings in each position from at least two separate and closely agreeing dilutions. At least two separate crystallizations were made from each separate species of blood, using a different animal for each crystallization.

In this investigation we used the Hüfner apparatus in which the thickness of layer of solution remains constant, and E is determined by measuring intensity of the remaining light. This is accomplished by means of an analyzing Nicol prism. It has been shown that the extinction coefficient E is equal to $-2 \log \cos \theta$ where θ is the angle of rotation just referred to. In order to obtain E , the angle of rotation θ necessary to produce equality in the upper and lower field is measured and E can then be computed. Bürker has computed a curve of coefficient E for angle of rotation from 60 to 80 degrees. Having determined the angle of rotation the values of E can be read directly from this curve. The curve used in this investigation appears

to be derived from the two published previously by Bürker (18). The experimental methods used in connection with the spectrophotometric work are fully described by Williamson (19) in a previous paper.

The balance of the hemoglobin in the weighing bottle was dried to constant weight over sulfuric acid. From the figures obtained for the moisture content of this sample, the dried weight of the sample used in the spectrophotometer was calculated. After we had determined the concentration of solution and the extinction coefficient, it was a simple matter to arrive at the

TABLE I.
Absorption Coefficients.

Animal.	A'_{o}	A_{o}
Dog.....	0.001191	0.001878
Ox.....	0.001187	0.001909
Cat.....	0.001137	0.001778
Chicken.....	0.001277	0.002002
Guinea pig.....	0.001180	0.001895
Rat.....	0.001130	0.001777
Sheep.....	0.001224	0.001934
Horse.....	0.001164	0.001846
Pig.....	0.001253	0.001988
Human.....	0.001179	0.001851

value of the absorption coefficient. Determinations were made on the hemoglobins of the dog, ox, cat, chicken, guinea pig, rat, sheep, horse, pig, and man. The results are shown in Table I.

DISCUSSION.

The figures obtained vary somewhat from those obtained by other investigators. The experimental conditions of this investigation were not precisely the same as those used by the former investigators. Our readings were made in spectral region 534.0 to 542.0 while those of Hüfner were made in the region 531.5 to 542.5 and those of Butterfield in the region 533.5 to 542.0. There were other slight differences in the conditions. Butterfield (9) has pointed out that minor differences in these conditions change the results appreciably. This may possibly explain the differences in results.

CONCLUSIONS.

From the results obtained in this experiment, it would appear that there is not sufficient difference in the absorption coefficients of the hemoglobin of various species to serve as a means of identification of the species. This finding confirms the conclusions of most of the previous investigators.

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FAT-SOLUBLE VITAMINE.*

III. THE COMPARATIVE NUTRITIVE VALUE OF WHITE AND YELLOW MAIZES.

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PLATE 2.

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In the preceding paper of this series (1) we emphasized the necessity of using caution when attempting to generalize in regard to the probable occurrence or non-occurrence of the fat-soluble vitamine in plant or animal tissues. In the data therein presented we indicated that tissues serving in the capacity of storage organs in the plant kingdom might nevertheless contain relatively large amounts of the fat-soluble vitamine. It was found to be an unwarranted procedure to conclude that only tissues of great activity contain large amounts of the fat-soluble vitamine as has been the tendency on the part of certain investigators in this field. In instances where no differences in biological activity were apparent nevertheless great variations in fat-soluble vitamine occurred. For instance, the carrot and the yellow sweet potato were found to contain so much of the fat-soluble vitamine that as a source of this dietary essential it was necessary to class them with leafy materials rather than with the cereal grains such as barley, wheat, oats, or even maize¹ which are acknowledged to contain relatively little of it. On the other hand, the mangel, sugar beet, red beet, parsnip, dasheen,

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¹ In American usage, corn is a specific term used for the designation of the grain *Zea mays*, but as in foreign English speaking countries this is not the case we have used the term maize.

rutabaga, and potato, though also admittedly storage organs, were found to be decidedly deficient in it. It was moreover emphasized that, though the conclusions in regard to the relative amounts of this vitamine occurring in the different materials that were investigated were undoubtedly correct, the values were not to be considered as absolute, as, for reasons unknown, differences in the amounts present were found to occur as indicated in the vitamine content of different samples of potatoes.

That such differences undoubtedly occurred we were impressed with, when 2 years ago we started to compare the fat-soluble vitamine content of cereal grains. From the statements prevalent in the literature at that time (2) we had received the impression that maize, as an example, was very poor in its content of this dietary constituent. We were therefore considerably surprised when in our experimental lots most of our rats on yellow maize—when this was suitably supplemented with vitamine-free protein and salts—continued to grow and ultimately reached maturity and maintained themselves in good condition with no signs of malnutrition; in fact not a single instance of xerophthalmia, which usually results secondarily from the fat-soluble vitamine deficiency, was recorded in these experimental groups. Obviously the case against maize as a fat-soluble vitamine deficient food was not so strong as the investigation of others had led us to believe.

As our data on the distribution of this dietary essential accumulated we were impressed with the fact that there appeared to be a remarkable coincidence in the occurrence of yellow plant pigments and resultant success in nutrition when all other requirements outside of the fat-soluble vitamine were known to be satisfied (3). For instance, both the carrot and sweet potato which are highly impregnated with yellow pigment were found to supplement successfully rations known to be deficient in this vitamine. Other roots not so pigmented were found impotent. Butter rich in pigment is very efficient and similarly oleo oils containing the pigment show a considerable fat-soluble vitamine content. Taking another example, we have in the case of the leafy parts of plants both the growth-promoting property and the appearance of yellow plant pigments associated, though here the yellow pigments are masked by the chlorophyll. At the present

time many such correlations have been made by us and shall later be presented in their proper connections. Suffice it to say that since these general premises have apparently justified abstract inferences in regard to the probable occurrence or absence of the fat-soluble vitamine on the color basis, it appeared probable that such correlations might be extended to that of the white and yellow maize kernels to which we shall here confine our consideration. It was recalled that at one time a fat-soluble vitamine deficiency in the ration of our stock animals was indicated and that at about this time white maize instead of yellow maize had been fed as a part of their ration. No etiological connection between the two, however, was established at this time.

In the data that follow, especially as will be seen from data graphically presented in the various charts, the surmisal of the differences in comparative dietary efficiency of white and yellow maize due to fat-soluble vitamine content in correlation with the occurrence of yellow pigments was substantiated. White maize, in every case where the experimental animals were dependent on it for their fat-soluble vitamine, produced absolute nutritional failure. On the other hand, yellow maize under similar conditions gave good and, even if not in most cases, sometimes normal results.

EXPERIMENTAL.

In general, the same experimental procedure that has almost become a standard in this laboratory in the last 2 years was used in these experiments. With a few exceptions, where the ration available imposed limitations, four young rats were kept in a group and fed as such but, as noted in the graphs, sometimes data with more than four animals are recorded. In these cases the experiments were repeated and the combined data presented in one chart. No record of the amounts of the rations consumed by the animals was kept other than that indicated by the total amounts of rations consumed which made no allowance for that wasted in the cages which often totals to a considerable quantity. While accurate consumption records are much to be desired and in many instances are even indispensable for validating certain conclusions it was felt that our general conclusions would not necessitate the additional labor which the keeping of such records

would entail, especially when on such a palatable food material as maize no critical variation in amounts consumed by the animals was to be expected.

In preparation for the ration, the maize was finely ground, and mixed with casein—which had previously been made vitamine-free by very dilute acetic acid extraction—and with salts. As maize at the level fed in the experiments carries plenty of the water-soluble vitamine for growth no supplementation with this vitamine was necessary to make growth possible when all the other dietary requirements were complied with. In the main, the experiments were confined to white and yellow maize, but in addition there was investigated a red maize carrying a white endosperm, a red maize carrying a yellow endosperm, and a variegated maize of red, yellow, white, and blue effects caused by variations in the distribution of these pigments between the pericarp, the aleurone layer, and the endosperm. The specific make-up of the rations is indicated in the various charts.

Fat-Soluble Vitamine in Yellow Maize.

The yellow maizes fed were such as were well recognized as distinct varieties grown in the corn-producing sections of the United States. They were represented by Reid's yellow dent, grown extensively in the corn belt; Murdock, grown in the northern corn belt; Golden Glow, Wisconsin No. 12, grown in central and southern Wisconsin, and an early yellow dent known as Wisconsin No. 8, grown in central and north central Wisconsin.

In Chart 1 are shown the curves of growth of nineteen young rats put on the yellow corn ration soon after weaning. As seen in the chart, in general, a very satisfactory degree of growth was observed with the rats on these different varieties of maize. It is true that there obtain some variations in the growth performance of the animals, but with the limited data available these differences are not to be associated offhand with variations in vitamine content, but rather are to be attributed to variations in the natural ability of the animal to grow irrespective of the character of the ration. It is not meant to infer that differences in vitamine content may not have obtained, but rather that these were not indicated unequivocally by the experimental technique employed. In a number of instances a poor showing in growth response or ultimate failure may require special mention.

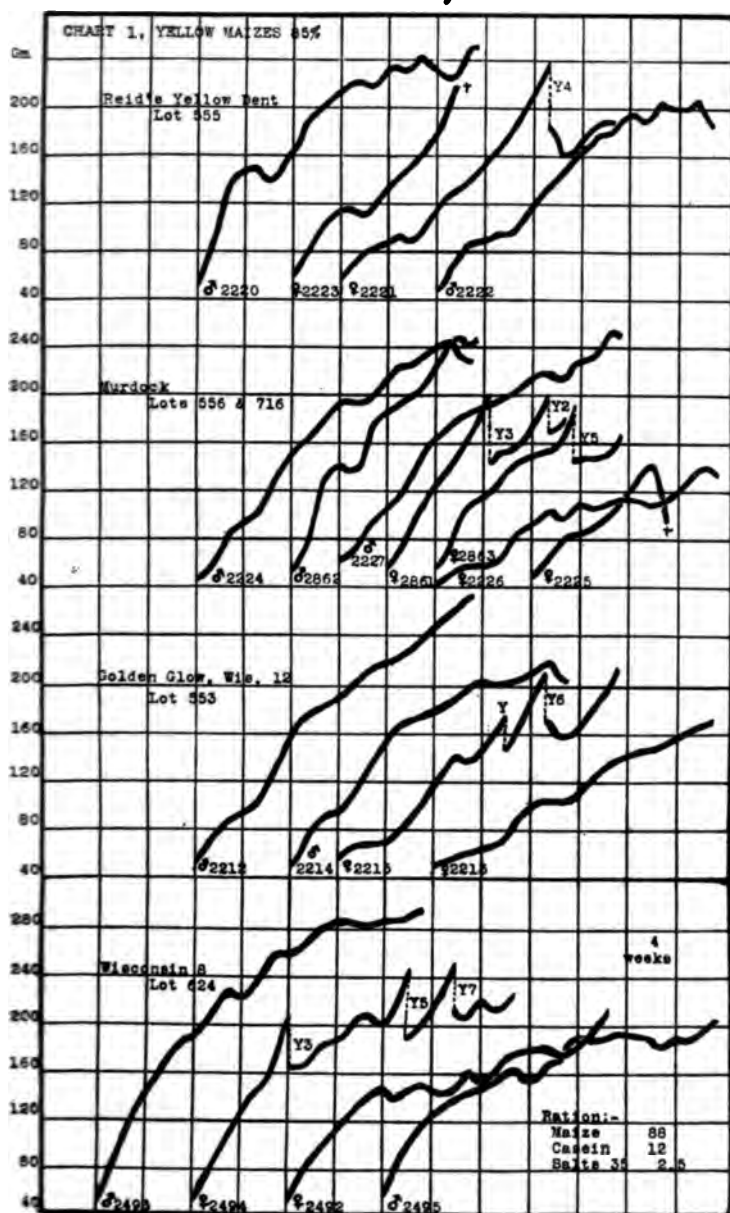


CHART 1.

In Lot 555 fed Reid's yellow dent, Rat 2223 died suddenly as a result of impaction of the stomach with large pieces of shavings gathered from the litter used in the cage. Her growth had been entirely normal. In Lots 556 and 716 fed the Murdock variety of yellow maize, Rats 2225 and 2226 gave poor results. Of these, the rapid failure of Rat 2225 is to be attributed to an infection localized in the generative organs, but the cause of the subnormal growth of Rat 2226 could not be ascertained. She appeared to be in good condition except for her weight. A similar statement can be made in regard to the case of Rat 2213 of the Golden Glow group, Lot 553. In Lot 624 fed Wisconsin No. 8, no special comments are necessary except with respect to Rat 2492 which failed to expel her fetus and necessitated our chloroforming her.

One fact which stands out dominantly is that of the nine pregnancies which occurred on yellow maize not one resulted in the production or rearing of young to the weaning stage. A statement of the individual conditions may bring this out forcibly.

In Lot 555, Rat 2221 gave birth to a litter of four young which were raised to an average weight of 20 gm. in 17 days. Though slightly undersized for their age they were in good condition until one day they were found, one dead and the others very lethargic and in apparent distress. The following day all were dead. In the Murdock group, Lot 716 produced three litters of young; of these Rat 2861 nursed one individual from her first litter to a weight of 30 gm. when it died, but none of her second litter lived longer than a few days; Rat 2863 nursed her young for 10 days when they too were found missing. On Wisconsin No. 12, Lot 553, Rat 2215 came near raising her second litter. While the first litter died on the day of birth the second litter containing six individuals was nursed for 3 weeks. Normal young in our colony average about 38 gm. at this age, but these six weighed only 111 gm. and died at the end of the 4th week. Wisconsin No. 8 supported reproduction no better in Lot 624. Here Rat 2494 lost her first two litters before they were a week old and her third when slightly over 3 weeks, though it had been reduced to four animals; yet the mother remained in excellent condition.

Offhand, this failure of reproduction is not to be associated with the fat-soluble vitamine relations, but the data here presented do not give us a basis for further comment.

In Chart 1 is indicated how, over a period of about 6 months, many rats on a ration in which yellow corn served as the sole source of fat-soluble vitamins grow at a rate which is to be considered normal. That growth can be continued over a longer period of time is shown in Chart 2. The rats of this group were fed a similar ration to those whose growth curves are shown in Chart 1 differing only in that to the 88 gm. of maize and the 12 of casein 0.75 gm. of sodium chloride, 4.43 gm. of calcium lactate, and 0.14 gm. of iron citrate were added instead of the 1.0 gm. of sodium chloride and 1.5 gm. of calcium carbonate. On this ration Rat 1707' and 1705' grew over a period of 13 months at

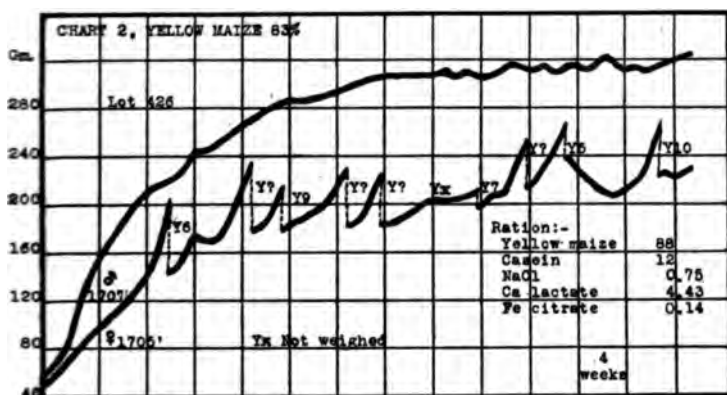


CHART 2.

rates which can be considered normal though it is probably true that the initial rapid increase of weight was not continued for so long a period as might have been expected for the average of our colony, but this may not have been entirely due to a limitation of the fat-soluble vitamins factor, as the same result has often been obtained on other supposedly good rations—including yellow maize—which we had supplemented with this vitamins. In the case of the female, no doubt her numerous pregnancies delayed her growth; yet she maintained herself in excellent condition. The male grew better, yet after the first 8 months of growth it became rather rough in appearance although there was no evidence of any cutaneous infections; its eyes remained large and bright and it continued to be sexually potent to the time

of writing. Unequivocal indications of a fat-soluble vitamine deficiency may then be said to have been totally absent.

It was noted in the discussion of the graphs in Chart 1 that absolute failure in the rearing of the young was the rule. This was duplicated in the first eight pregnancies of this female, in fact all the young were found dead shortly after birth or else were found missing before any record of their number or weight had been obtained. Our data on this are complete with one exception where by an oversight with a change in the laboratory staff the rat was not weighed or closely observed for 3 weeks, and so it is not known how long that litter lived. To provide more favorable conditions for the rearing of the young, the young of the ninth pregnancy were reduced in number to three animals shortly after birth. These were reared rapidly, averaging 36 gm. in weight at the end of 3 weeks and 45 gm. at the end of 4 weeks which is a rate of growth that can be considered normal. With the tenth litter reduced to four animals this performance was duplicated. We have therefore secured information showing that it is possible to secure the rearing of some young on a ration where yellow maize is the sole source of the fat-soluble vitamine. In instances where lactating mothers were transferred when mature from our standard stock ration to the corn ration fed in this lot, no difficulty in the rearing of young has been experienced, in fact, both the experimental Rats 1707' and 1705', whose history was just discussed, were members of a litter brought up under these conditions. It is thus indicated that, when rats make their growth on such a ration and have later the added demands of lactation imposed on them, they ~~can~~ not meet the requirements of the situation as they can when they have been reared on a better ration. But it is not to be inferred that the fat-soluble vitamine is necessarily the limiting factor. Furthermore, it remains to be seen if the fact that the young of the earlier pregnancies were not reared, while those of the later were, has any special significance.

Fat-Soluble Vitamine Content of White Maizes.

When young rats at an entirely comparable period in their development to those shown in Charts 1 and 2 are put on white maize supplemented with purified proteins and salts, failure in

growth and even in maintenance of life rapidly ensues. This is shown in Chart 3. In the dissection of these data we are limited in the conclusions that we can draw by the fact that differences in growth performances can, to a certain extent—not determined—be attributed to variations in the growth impulse of the animals. To a very great degree these differences should be erased by the method we used in the selection of our animals, because in this procedure animals are selected for differ-

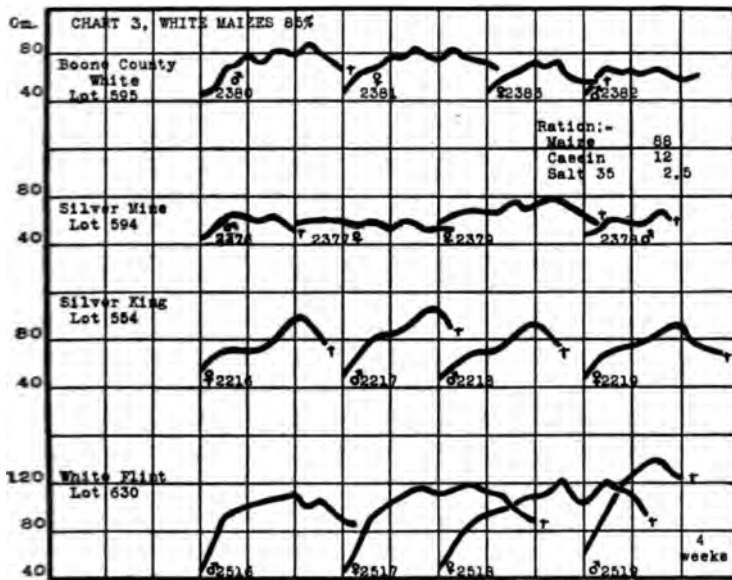


CHART 3.

ences in coat patterns from a group of 50 to 100 of suitable age and size gathered in one cage. In this way the chances are that few if any groups of four consisted of individuals from only one litter and therefore they would be unlikely to have similar inherited tendencies. With this in mind it appears rather remarkable that there should obtain such similarity in the character of the curves of growth of the animals within the groups and such distinguishing features—at least in the amount of growth—among the different groups if differences in nutritive value did not obtain. We are inclined to believe that the better

growth performance of the White Flint group was due to a greater fat-soluble vitamine content. This is noteworthy especially as the White Flint maize though a commercial white corn was not a "dead" white in comparison with the Silver Mine variety. Some allowance must be made for the difference in appearance bound to result in a flint maize as compared with the dent, yet making this allowance this variety appeared to have a distinct tinge of yellow both in the grain and as a meal. We do not care to stress this point reserving our conclusions until appearances are verified by chemical examination, but with the evidence given by the growth curves we believe it highly probable that there are differences in the vitamine content of the so called white maizes corresponding to their degree of yellow pigmentation. Certain it is that all these varieties are far different in their fat-soluble vitamine content from those of the yellow variety.

In the four lots receiving white maize as the sole source of the fat-soluble vitamine in their ration we had abundant demonstration of its deficiency in this constituent by the observed symptom complex which while not entirely specific yet in a large group of animals gives indications of the exact nutritive status with a fair degree of certainty. First in importance there is the failure of growth ending in failure of maintenance of life; second there is the often occurring inflammation of the eyes, a conjunctivitis or xerophthalmia as pointed out by Osborne and Mendel; and thirdly there may prevail the general condition of malnutrition of the skin as indicated by encrustation of the ears, warts on the nose, infection on the tail and feet, and even sores on the body itself. All these occur singly or collectively, but as they are not absolutely specific they are to be considered suggestive symptoms instead. In the group of rats all failed to grow and no doubt all would have died if the experiments had been continued a short time longer as twelve of the sixteen animals died during the time of observation. Of the total, nine had contracted an infection in one or both eyes and three had cutaneous infections.

*Comparative Fat-Soluble Vitamine Content of White and
Yellow Maizes.*

In order to bring out the limitations in the amounts of fat-soluble vitamine in yellow and white maize more concretely than the previous lots had indicated two experimental groups were fed these grains in which 50 instead of 88 parts of the ration—exclusive of the added salts—consisted of maize (Chart 4). The results are obvious. They indicate that with a reduction in the amount of white maize in the ration growth is little worse than with some of the white maizes at the higher level. Unfortunately the white maize fed in this lot was not of any standard variety.

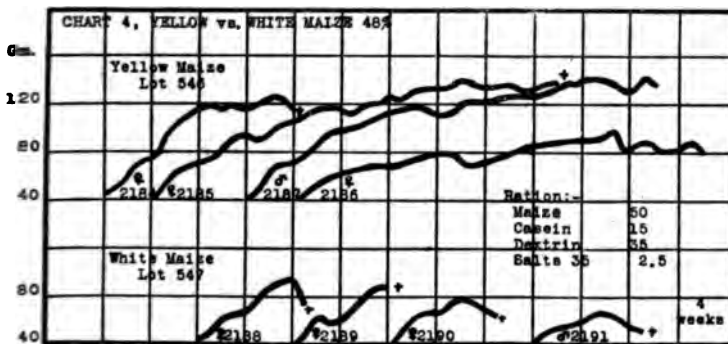


CHART 4.

but was obtained on the local market as a distinctly white variety so direct comparisons are not possible, but apparently it seems safe to infer that certain white maizes contain so little of the fat-soluble vitamine that as a source of this dietary essential they can be considered valueless. This is the only conclusion that can be arrived at when it is seen that a reduction in the amount of white maize in the ration causes so little difference in the resultant growth. Such growth as is observed must be attributed to the fat-soluble vitamine stored in the body of the animal. Three of the animals, viz. Rats 2188, 2190, and 2191, developed xerophthalmia, but Rat 2189 died without any visible signs of infection.

With the yellow maize a reduction of the amount in the ration from 85 to 48 per cent gives a definite corresponding reduction in the amount of growth. Growth was possible at only about one-

half the normal rate from which we may infer—as the reduction in maize and therefore vitamine content was approximately 40 per cent—that yellow maize when fed in maximum amounts contains just sufficient fat-soluble vitamine to make normal growth possible.

Fat-Soluble Vitamine Content of Red Maizes.

With a relation between the occurrence of the yellow pigment and the growth-promoting property associated with the fat-soluble vitamine established in the case of yellow maize, it re-

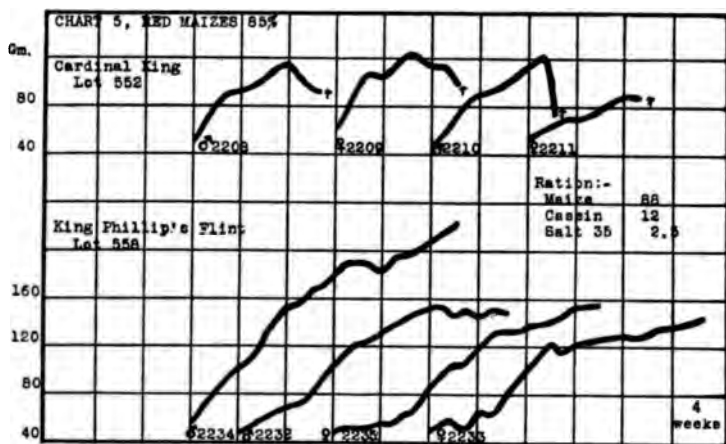


CHART 5.

mained to determine what the situation might be in the case of maizes of other colors. This opportunity presented itself with two varieties known respectively as Cardinal King and King Phillip's Flint. It is seen at a glance in Chart 5 that failure on the former ensued rapidly while on the latter growth continued at a subnormal rate for 22 weeks when the experiment was terminated. The difference can again be correlated with reference to the presence of yellow pigments for while both varieties were red, the Cardinal King had a white endosperm while the King Phillip's Flint had a yellow endosperm; the former has no visible yellow pigments, while the latter has considerable.

The rats on Cardinal King in Lot 552 succumbed to the fat-soluble vitamine deficiency very rapidly, all dying in less than 12 weeks after they had been put on the ration. Their behavior was very similar to those on the white maize, Chart 3. All except Rat 2211 contracted xerophthalmia.

On King Phillip's Flint when the experiment was discontinued—almost 23 weeks after its inauguration—all the animals were rated as being in good condition giving no evidence of eye infections or cutaneous malnutrition.

Fat-Soluble Vitamine Content of Variegated Maize.

Maize, as indicated by the innumerable so called varieties which are grown in the United States, offers many opportunities for the intermingling of characters not only in habit of growth, but also in color. In variegated maize many color characteristics

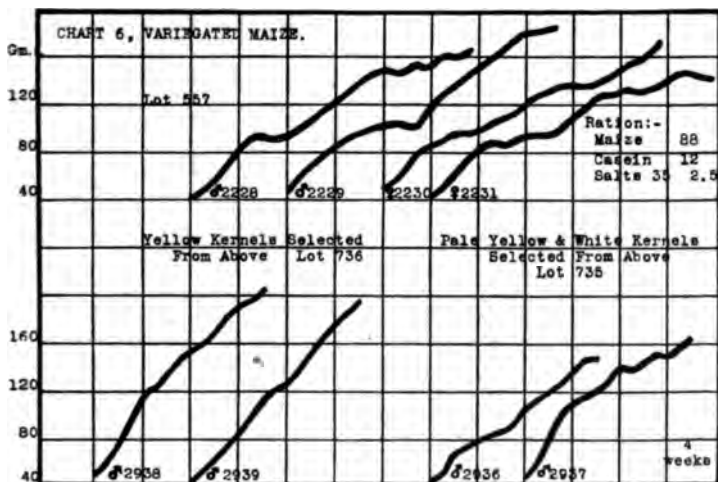


CHART 6.

may be associated in the same kernel or in different kernels on the same cob. Such a maize was the sample whose fat-soluble vitamine content was demonstrated in Lot 557, Chart 6. All the rats grew a little better than one-half the normal rate and were in good condition when the experiment was discontinued.

It is noteworthy that there again the maize fed had a very large proportion of its kernels bearing varying amounts of yellow pigment especially in the endosperm, but some of the kernels were distinctly white and others, when pigmented, had a white endosperm.

It remained to establish how closely the fat-soluble vitamine was associated with the yellow pigments in this sample of maize where color characteristics were so closely interwoven that from the hereditary standpoint it was impossible to predict what the color distribution in the crop from any one kernel would be. To determine this, the seeds predominatingly yellow were selected by hand from those pale yellow or more nearly white. The results are shown in Lots 735 and 736. As before, the intimate association of yellow pigment and the property attributed to the occurrence of the fat-soluble vitamine obtains. We believe all our data substantiate this thesis beyond a doubt.

Supplementation of White Maize with Fat-Soluble Vitamine.

As all our rations, outside of the fat-soluble vitamine, contained all the generally recognized required ingredients of a complete ration and as many of our animals on the white maize rations consistently, singly or collectively, gave all the symptoms of a fat-soluble vitamine deficiency there remains no alternative but to conclude that the failure of rats to grow on white maize rations is due to a lack of fat-soluble vitamine. It seems rather remarkable that these differences in the nutritive value of the maizes should not have been generally recognized especially when it is considered that both are used in large amounts for feeding animals. As a matter of fact it has been reported by some stockmen that yellow maize is superior to white maize, but again by others it has been as stoutly denied. As chemical analysis for determining nutritive values of feeds has indicated no differences in digestible fat, protein, nitrogen-free extract, fiber, and ash, the entire matter has been given little attention in nutrition fields, as the limitations of such analysis have been little realized (4). Chart 7 indicates, if data obtained with rats are applicable to other animals, that from the practical standpoint this difference of opinion may be based on justifiable grounds. Ordi-

narily materials rich in the fat-soluble vitamine are consumed by man, as well as by animals, in sufficient amounts to supplement the fat-soluble vitamine-poor foods efficiently. But under restricted feeding conditions the situation is far different. It ap-

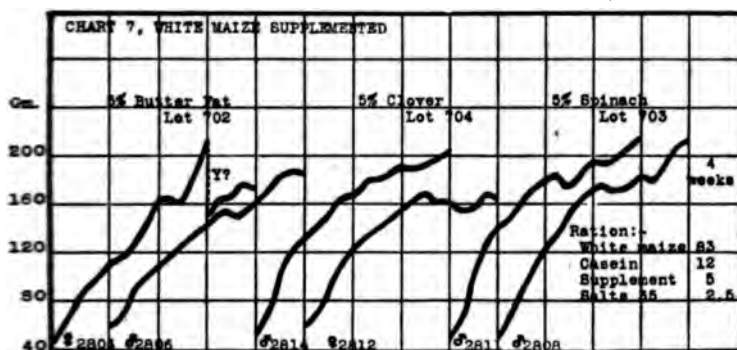


CHART 7.

pears a curious reflection on human instinct that in the United States and Mexico white maize is preferred to yellow maize as a major constituent of the diet, especially when it is considered that this is the case in sections where restricted rations are common.

CONCLUSIONS.

The occurrence of yellow pigment and the growth-promoting property attributed to the presence of the fat-soluble vitamine seem to be intimately associated in the maize kernel.

Yellow maize contains enough of the fat-soluble vitamine to allow growth at the normal rate to take place in the rat; reproduction was possible, but usually was a failure; maintenance without premature signs of senility was also observed.

White maize does not contain any demonstrable amounts of the fat-soluble vitamine. In attempts to have it serve as the source of fat-soluble vitamine it seldom allows rats to remain alive longer than 3 months. One commercially so called white maize allowed a certain amount of growth to take place, but close inspection indicated the presence of some yellow pigment.

Red maize with a white endosperm free from yellow pigment gave the same results as white maize; red maize with a yellow

endosperm gave results though not of the same degree, yet approximately that of yellow maize.

A variegated maize of red, yellow, white, and blue effects due to variation in color distribution in different kernels between pericarp, aleurone layer, and endosperm gave results intermediate between those obtained with yellow and white maize; in correlation most of the kernels had yellow endosperms, the rest had white endosperms.

When from the variegated maize the yellow kernels were selected from those that were white or a very pale yellow and fed to young rats, the performance of growth was distinctly better on the former.

In a mixed diet containing maize the fat-soluble vitamine deficiency of white maize is easily taken care of by the supplementary action of other foods rich in this dietary essential.

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EXPLANATION OF PLATE 2.

FIG. 1. Rat 2215, female, after having been fed for 78 days on a ration in which all the fat-soluble vitamine was supplied by 85 per cent of yellow maize. When started on the ration it weighed 59 gm.; when photographed 137 gm. Note its good nutritive condition. For weight record see Chart 1, Lot 553.

FIG. 2. White Rat 2377, female, and hooded Rat 2379, female, after having been fed for 92 days on a ration in which all the fat-soluble vitamine was supplied by 85 per cent of white maize. For weight records see Chart 3, Lot 594. The white individual originally weighed 47 gm.; when photographed it weighed 53 gm. Its ears were infected, but its eyes were normal.

The hooded rat originally weighed 57 gm.; when photographed it weighed 60 gm. Note the encrustation of the ears and the inflamed eye. It died 2 days later.



FIG. 1.



FIG. 2.

(Steenbock and Boutwell: Fat-Soluble Vitamins. III.)



PROTEIN REQUIREMENT OF MAINTENANCE IN MAN AND THE NUTRITIVE EFFICIENCY OF BREAD PROTEIN.

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In connection with our studies of nutritive requirements and of the efficiency of the proteins of the cereal grains in the maintenance metabolism of man¹ we have taken occasion to bring together the results of such of the earlier experiments upon the protein requirement of maintenance as seem to lend themselves to direct quantitative comparison. The purpose of the present paper is to present as concisely as possible the net result of this study of the literature together with the data of our own experiments upon a dietary in which nearly all the nitrogen was taken in the form of wheat bread.

An attempt to determine the protein requirement of maintenance, even for any one kind of protein, would ideally involve adherence to an otherwise uniform diet with complete findings of nitrogen intake and output and with periodical increase or decrease of protein consumption without alteration of the energy supply, until it is shown that the body can maintain itself in nitrogen equilibrium on a certain amount of protein, of the kind or kinds which the diet in question furnishes, and not on any smaller amount. The recorded investigations which meet all these conditions are not sufficiently numerous, nor do they cover a sufficient number of men and women or a sufficient range of foods to form a satisfactory basis for any general deduction as to

¹ Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. Sherman, H. C., Wheeler, L., and Yates, A. B., *ibid.*, 1918, xxxiv, 383. Sherman, H. C., and Winters, J. C., *ibid.*, 1918, xxxv, 307. Sherman, H. C., Winters, J. C., and Phillips, V., *ibid.*, 1919, xxxix, 53.

the amount of protein required in normal adult maintenance. In attempting such a deduction, proper consideration must also be given to the much larger number of experiments in which the balance of intake and output of nitrogen has been determined with subjects living on low protein diets under conditions so arranged and controlled as to make it probable that the rate of protein metabolism was at least approximating the minimum at which normal equilibrium could be maintained.

Probably the best present indication of the normal protein or nitrogen requirement is to be obtained by averaging the observed nitrogen output in all available experiments in which the intake appears to have been barely sufficient or not quite sufficient to result in equilibrium of intake and output.

Since the protein minimum thus determined is influenced by the protein-sparing action of carbohydrates and fats, the results will be comparable, and will bear directly upon the practical problems of protein requirement in food economics, only in those cases in which the energy value of the experimental ration is at least approximately adjusted to the energy requirement of the subject.

Many of the published experiments which were designed to test the amounts of protein required in normal nutrition are now seen to have given misleadingly high results either because the food was reduced as a whole, thus creating a deficit in the energy intake which interfered with the economical use of the protein, or because, influenced by past overestimates of the protein requirement, the experimenters did not sufficiently reduce the amount of protein in the food to make a real test of the minimum on which equilibrium could be established and maintained.

Given a proper adjustment of the energy value and a sufficiently restricted proportion of protein in the experimental ration, the question arises as to how closely the nitrogen output must agree with the intake in order that it may be accepted as indicating the approximate requirement of the subject under the conditions of the experiment.

A plus balance or exact equilibrium of nitrogen intake and output always suggests the question whether equilibrium might not have been again established if the nitrogen intake had been further reduced, in which case the given result was obviously higher than the amount actually required. Whether the observed

output is such an overestimate or is a fair approximation to the amount really required for equilibrium can often be judged with a fair degree of confidence from a detailed and critical study of the investigation as a whole. At any rate, when there is nitrogen equilibrium on a low protein intake it seems safe to conclude that the diet is at least meeting all the requirements of the protein metabolism of maintenance.

Also, when the nitrogen output is only slightly greater than the intake it seems permissible to regard the output as approximating the amount on which equilibrium could have been maintained; for while it is conceivable that a small loss of body nitrogen may represent a real inadequacy of the intake, perhaps as regards some particular amino-acid, yet it is usually much more probable that a small negative balance means simply that the body has not yet completed the adjustment of its output to its intake and that a continuation of the experiment would have shown a lower output. Thus while it is possible that the output may sometimes be smaller than the amount which would be required for equilibrium, yet from the large amount of evidence now available it seems fairly certain that any error of this sort can have but very slight influence upon the final average, and in all probability is more than offset by the tendency toward high results introduced through including in the average a considerable number of experiments in which the protein of the diet was not reduced to a sufficient extent, and for a long enough time, to show the real minimum at which normal equilibrium could be maintained.

In order to minimize the personal equation in our interpretation of the work of others, we have uniformly excluded from Table I all experiments showing a loss of body nitrogen greater than 1 gm. per day even though in some cases this necessitated the omission of data of undoubted value.

There remained 109 experiments belonging to twenty-five different investigations and including 67 experiments upon twenty-nine men and forty-two experiments upon eight women subjects. For convenience of comparison the total nitrogen output per day (urine and feces) is computed to a basis of 70 kilos of body weight and multiplied by 6.25 to express the corresponding amount of protein which is tabulated as the "indicated protein requirement" in each of the 109 experiments as shown in Table I.

TABLE I.*

Indicated Protein Requirements per 70 Kilos of Body Weight.

Experiment No.	Indicated protein requirement.	Experiment No.	Indicated protein requirement.	Experiment No.	Indicated protein requirement.	Experiment No.	Indicated protein requirement.	Experiment No.	Indicated protein requirement.
	gm.		gm.		gm.		gm.		gm.
1	39	23	45	45	31	67	56	89	37
2	38	24	49	46	37	68	57	90	38
3	45	25	56	47	45	69	60	91	39
4	33	26	47	48	50	70	57	92	39
5	30	27	62	49	39	71	54	93	39
6	65	28	61	50	52	72	51	94	43
7	61	29	55	51	52	73	52	95	40
8	41	30	53	52	42	74	54	96	39
9	50	31	53	53	42	75	54	97	43
10	43	32	61	54	24	76	53	98	38
11	37	33	40	55	21	77	53	99	37
12	29	34	56	56	33	78	44	100	33
13	59	35	43	57	32	79	45	101	37
14	57	36	42	58	32	80	46	102	41
15	49	37	41	59	39	81	46	103	51
16	42	38	39	60	50	82	42	104	44
17	50	39	48	61	58	83	41	105	42
18	48	40	46	62	37	84	36	106	32
19	49	41	38	63	41	85	38	107	35
20	54	42	47	64	44	86	39	108	34
21	36	43	36	65	59	87	39	109	32
22	47	44	37	66	60	88	38		
Average.....									44.4

* Experiments 1 and 2, Hirschfeld, F., *Arch. ges. Physiol.*, 1887, xli, 533. No. 3, Hirschfeld, F., *Virchows Arch. path. Anat.*, 1888, cxiv, 301. Nos. 4 and 5, Klemperer, G., *Z. klin. Med.*, 1889, xvi, 550. Nos. 6 and 7, Viot, C., *Z. Biol.*, 1889, xxv, 232. No. 8, Pechsel, Eiweissbedarf des gesunden Mensch., Dissertation, Berlin, 1890 (*U. S. Dept. Agric., Bull.* 45, 1898). No. 9, Lapique, L., *Arch. Physiol.*, 1894, Series 5, vi, 596. Nos. 10 and 11, Sivén, V. O., *Skand. Arch. Physiol.*, 1900, x, 91. No. 12, Sivén, V. O., *ibid.*, 1901, xi, 308. No. 13, Albu, *Z. klin. Med.*, 1901, xliii, 75. Nos. 14 to 20, Jaffe, M. E., *U. S. Dept. Agric. Bull.*, 132, 1902. No. 21, Caspari, W., and Glaessner, K., *Z. diät. physik. Therap.*, 1903, vii, 475. Nos. 22 to 33, Chittenden, R. H., *Physiological economy in nutrition*, New York, 1904. No. 34, Aron, H., and Hocson, F., *Biochem. Z.*, 1911, xxxii, 189. Nos. 35 to 38, Hindhede, M., *Skand. Arch. Physiol.*, 1912, xxvii, 277; Nos. 39 to 46, 1913, xxx, 97; Nos. 47 to 55, 1914, xxxi, 259. Nos. 56 to 61, Abderhalden, E.,

The 67 experiments upon men show an average "indicated protein requirement" of 0.633 gm. per kilo, while the 42 experiments with women average 0.637 gm. per kilo of body weight. It seems unnecessary, therefore, to distinguish between the sexes in this discussion since we are dealing with data calculated to a uniform basis of body weight.

The general average of the 109 experiments shows an indicated requirement of 0.635 gm. of protein per kilo of body weight, or 44.4 gm. per "average man" of 70 kilos, per day. Two considerations, one favorable the other unfavorable, should be kept in mind in attempting to judge the scientific value of this average. On the one hand, it represents a very large amount of work in several different laboratories and on many subjects, both men and women, so that errors due to individual peculiarities of subjects, diets or conditions, or the personal equation of the investigator are minimized. On the other hand, the data of individual experiments are rather divergent, ranging from a minimum of 21 gm. to a maximum of 65 gm. per 70 kilos. It should perhaps be noted that the very large majority of these experiments (94 in 109) yield values within the limits of 29 to 56 gm. per 70 kilos, and that these more concordant data taken separately show an indicated protein requirement averaging 42.8 gm. per 70 kilos based on 94 experiments covering thirty-four subjects (twenty-six men and eight women) studied in twenty-three different investigations. If we go a step further in rejecting the extremes, we find within the limits of 30 to 50 gm. per 70 kilos per day, 76 experiments belonging to nineteen different investigations, including twenty-four subjects (twenty men and four women) and averaging 40.6 gm. per man of 70 kilos, or 0.58 gm. per kilo of body weight per day.

Ewald, G., Fodor, A., Röse, C., *Arch. ges. Physiol.*, 1914-15, clx, 511. Nos. 62 to 64, Rose, M. S., and Cooper, L. E., *J. Biol. Chem.*, 1917, xxx, 201. Nos. 65 to 72, Sherman, H. C., Gillett, L. H., and Pope, H. M., *ibid.*, 1918, xxxiv, 373. Nos. 73 to 77, Sherman, H. C., Wheeler, L., and Yates, A. B., *ibid.*, 1918, xxxiv, 383. Nos. 78 to 93, Sherman, H. C., and Winters, J. C., *ibid.*, 1918, xxxv, 301. Nos. 94 to 102, Sherman, H. C., Winters, J. C., and Phillips, V., *ibid.*, 1919, xxxix, 53. Nos. 103 and 104, Sherman, H. C., and Rose, A. R., not previously published. No. 105, Sherman, H. C., and Beegle, F. M., not previously published. Nos. 106 to 109, Sherman, H. C., and Osterberg, E., not previously published.

Thus according as we include all 109, or 94, or 76 experiments in the average it becomes respectively 0.635, 0.61, or 0.58 gm. per kilo, or in round numbers 45, 43, or 41 gm. per man of 70 kilos per day. It will be noted that the more critical the selection of the experiments to be included in the average the lower the indicated protein requirement becomes. There would appear to be a smaller probable error in estimating the average protein requirement at about 0.6 gm. per kilo, or 42 gm. per 70 kilos, per day, than at any higher figure. It should also be noted that the final data of nearly all the more extended and the more closely controlled investigations fall below rather than above this average.

From a general review of all the experiments included in this compilation it appears to us that the chief cause of variation was the differing duration of the investigations and the differing extent to which the subject had accustomed himself to a low protein diet. In the work thus far published this seems to have been a more influential factor in determining the amount of protein apparently required for maintenance in the human adult than was the nature of the protein fed.

We have previously shown that the protein of rations consisting essentially of corn (maize) meal or oatmeal supplemented by only small amounts of milk, the latter furnishing but one-tenth to one-fifth of the food protein, may be fully as efficient in the maintenance metabolism of man as the average protein of ordinary mixed diets has been found to be in the work of previous investigators. Because of the large extent to which the majority of the people of this country and of Europe depend upon bread as their chief source of protein as well as energy, it seems well to give in this connection the data of our chief series of experiments in which ordinary (white) wheat bread furnished almost all the protein of the food.

Experiments upon the Efficiency of Bread Protein in Maintenance Metabolism.—The subject, E. O., a man weighing 80 kilos, took on each of 15 successive days a ration consisting of 400 gm. of bread, 150 (or, during the last 6 days, 200) gm. of butter, and 300 gm. of apple. The 15 days were divided into five experimental periods of 3 days each, the foods and feces being collected, sampled, and analyzed separately for each of these periods. Urine was collected and analyzed separately for each 24 hours. The work

thus constitutes a series of five complete balance experiments each of 3 days duration and following each other without intermission. The data of nitrogen intake and output are shown in Tables II and III.

In these experiments bread furnished over 95 per cent of the protein consumed, yet allowing the first 3 days for adjustment it will be seen that practical equilibrium was maintained on an intake of a little less than 0.5 gm. of protein per kilo of body weight per day. Thus the protein of wheat bread showed as high an efficiency in the maintenance metabolism of man as would be expected of the protein of mixed diet in general. The bread was ordinary white bread purchased from a New York City

TABLE II.

Food Eaten with Daily Intake of Nitrogen from Each Source. Experiments with E. O., Weight 80 Kilos.

Kind of food.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
	gm.	gm.	gm.	gm.	gm.
Bread.....	400	400	400	400	400
Butter.....	150	150	150	200	200
Apple.....	300	300	300	300	300
Nitrogen from bread.....	5.77	5.87	5.76	5.71	5.72
“ “ butter.....	0.13	0.13	0.13	0.18	0.18
“ “ apple.....	0.11	0.09	0.11	0.11	0.12
Total per day.....	6.01	6.09	6.00	6.00	6.02
Protein per day.....	38	38	38	38	38

bakery. Probably the customary small amount of milk was used in making the bread—exactly how much or in what form we were not permitted to ascertain. That the bread did not contain any unusual proportion of milk or milk powder was shown by determining its content of phosphorus and calcium as well as of nitrogen.

Thus it does not seem necessary to discriminate against bread protein as compared with the protein of staple foods in general in so far as the requirements of adult maintenance alone are concerned. This result is in opposition to the claims of Karl Thomas but is in accordance with the findings of Hindhede and of Morgan

and Hintze in their experiments upon man, as well as with the work of this laboratory on the efficiency of maize and oat proteins in adult human nutrition, and is in harmony with the findings both of Osborne and Mendel and of McCollum and his

TABLE III.
Daily Intake and Output of Nitrogen. Experiments with E. O.

Experiment No.	Day.	Body weight.	Nitrogen.				
			In food.	In urine.	In feces.	Output.	Balance.
1	1st 2nd 3rd	kg. 81.0	gm.	gm.	gm.	gm.	
			6.0	6.6			
			6.0	6.6			
			6.0	6.6			
Average.....			6.0	6.6	0.7	7.3	-1.3
2	4th 5th 6th	80.5	6.1	5.5			
			6.1	4.3			
			6.1	5.1			
			Average.....			6.1	5.0
3	7th 8th 9th	80.5	6.0	5.2			
			6.0	5.4			
			6.0	5.7			
			Average.....			6.0	5.4
4	10th 11th 12th	80.5	6.0	6.0			
			6.0	4.5			
			6.0	5.2			
			Average.....			6.0	5.2
5	13th 14th 15th	80.3	6.0	4.5			
			6.0	5.1			
		79.8	6.0	4.7			
		Average.....			6.0	4.8	1.0

associates that the proteins of cereal grains need only be fed in very moderate quantity to provide for the maintenance of body weight in adult rats. Thus Osborne and Mendel found the body weight maintained by food mixtures which contained only

5 to 7 per cent of the proteins of the whole wheat kernel,² which, as these food mixtures contained about 25 per cent fat, would mean that wheat protein sufficient to furnish from 5 to 6 per cent of the total calories of the food consumed was sufficient for maintenance. McCollum³ gives 4.5 per cent of oat protein or 6 per cent of wheat or maize protein as the proportion necessary to maintain body weight in adult rats. In McCollum's food mixtures the percentage of protein by weight would be about the percentage of protein calories in the total calories of the food.

Thus the feeding experiments upon rats are fairly consistent in indicating that the amount of grain protein required for adult maintenance (in this case judged by body weight) is about 6 per cent of the total food calories. Turning to the experiments upon the maintenance of nitrogen equilibrium in man, we see from the data above cited an indicated average protein requirement of about 0.6 gm. of protein per kilo of body weight per day. This corresponds to 2.4 protein calories, or 6 per cent of the 40 calories per kilo which is commonly accepted as a fair average energy requirement for moderately active men and women. Thus it would appear that the protein minimum for normal adult maintenance is very similar for man and the rat if the protein is stated in terms of the total calories of food consumed. It does not necessarily follow that the protein requirement of growth will be proportionately as high for the child as for the young rat. The percentage rate of growth is very much greater in the young rat than in the child and healthy children are usually more active than the average of young rats at corresponding stages of growth. From these facts it is to be expected that the requirement for protein relative to calories will be greater in the case of the young rat than of the child at a corresponding stage of development. Relative to body weight, the protein requirement of growth is of course higher than that of maintenance, in either species. Of at least equal importance is the fact that the amino-acid make-up of the food protein is a more prominent factor in the problem of growth than in that of maintenance. In connection with the well known work of Hopkins, of Osborne and Mendel, and of

² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

³ McCollum, E. V., *Newer knowledge of nutrition*, New York, 1918, 75.

McCollum this fact has been discussed in terms of the functions of individual amino-acids both as tissue constituents and as possible precursors of hormones and it has been suggested that the protein requirement of maintenance may actually be a requirement for certain amino-acids for transformation into hormones, that the repair processes of maintenance may not involve the disruption and resynthesis of entire protein molecules, or that some of the amino-acids may be used over again in the repair processes. It may also be helpful to think of the protein metabolism not only in terms of building and repair, but also of maintaining the equilibrium which exists between proteins and amino-acids in the cells of the animal tissues. In such a cell there is a constant tendency toward removal of amino-acids by deamination, offset by the inflow of amino-acids derived from food protein and brought to the tissue cell by the blood stream. Concentration of any of the amino-acids into which tissue proteins tend to be hydrolyzed may therefore be expected to help in pushing the reaction, **amino-acids \rightleftharpoons protein**, toward the right; in other words, *any* of these amino-acids will function in the *maintenance* of body protein, whereas for the synthesis of new protein as in *growth*, *all* the amino-acids would be needed. Hence it is quite reasonable that proteins of very different efficiency for growth may show much more nearly equal efficiency in the normal maintenance nutrition of adults.

It is well known that as a group the proteins of the cereal grains are less rich in certain amino-acids essential to animal tissue, notably lysine and tryptophane, than are several other of the food proteins; and it is reasonable to expect that such differences in chemical structure among proteins imply corresponding differences in nutritive efficiency. But deficiencies established by experiments with isolated proteins do not necessarily imply corresponding deficiencies in the nutritive value of the natural mixtures of proteins found in our ordinary articles of food. Most experiments designed to compare the nutritive efficiencies of the proteins of different foods have been performed upon laboratory animals during growth, since the nutritive requirements of growth naturally tend to accentuate the differences of food value among proteins which the experiments are designed to discover or demonstrate. Largely for the same reason, however, there is

danger that generalizations from such experiments may lead to exaggerated impressions. On the other hand, we would avoid giving an exaggerated impression in the other direction when we point out that any amino-acid such as results from hydrolysis of body protein may be expected to function in the maintenance metabolism. It is not probable that a molecule of tissue protein is hydrolyzed into a great number of molecules of amino-acids, of for example, fifteen different kinds, in a single step. Autolysis experiments indicate rather that there are successive splittings more or less similar to those which occur in digestive proteolysis, with liberation of amino-acids throughout the process as well as at the end. Any one amino-acid, then, could be expected to check the process only at the point at which that amino-acid would be liberated from the catabolizing protein molecule. If, however, we feed a protein which furnishes considerable amounts of, for example, twelve out of fifteen of the amino-acids in question, then even though the three which are lacking are strictly essential there will still be twelve chances in fifteen of checking the catabolism of the body protein at an early stage, with corresponding efficiency of the incomplete food protein in the protein metabolism of maintenance.

The efficiencies actually found in our typical experiments in which the protein consumed was almost entirely in the form of ordinary wheat bread, or of hard bread made from corn-meal or oatmeal, may be summarized as follows.

Subject R, a man of 80 kilos, established nitrogen equilibrium on a diet of 400 gm. of white wheat bread, 200 gm. of butter, and 300 gm. of apple per day furnishing in all 2,700 calories and 6.0 gm. of nitrogen of which latter 96 per cent was in the form of bread. Thus 37.5 gm. of protein for a man of 80 kilos, corresponding to 33 gm. for a man of average weight (70 kilos), sufficed for the maintenance of normal equilibrium.

Subject O, a woman of 55 kilos, maintained approximate equilibrium (losing less than 0.5 gm. of nitrogen per day) on a diet of corn-meal, butter, sugar, and apple which furnished 2,030 calories and 4.36 gm. of nitrogen per day, all the latter being maize protein except the small amount furnished by the apple. The actual intake of protein was 27 gm. per day. This corresponds to 34 gm. of protein per 70 kilos of body weight per

day. When 100 gm. of milk were added to the diet, making the actual intake of protein 30 gm. (equivalent to 37 gm. per 70 kilos) of which 88 per cent was from corn-meal, 10 per cent from milk, and 2 per cent from apple, the protein supply proved more than sufficient and the subject stored nitrogen. To compare these data with those of the preceding series would somewhat unduly favor the wheat in the comparison since Subject R was considerably older than Subject O and carried a larger proportion of adipose tissue. Under the circumstances, therefore, it seems proper to conclude that the corn protein has shown itself equally efficient with that of wheat in these experiments.

The experiments upon the efficiency of the proteins in oatmeal were carried out with two young women as subjects. The first, Subject O, weight 55 kilos, was the same who had served in the corn-meal experiments just described. The second, Subject P, weighed 67 kilos. Corn-starch was mixed with the oatmeal in such proportions that the cereal part of the diet should furnish practically the same proportions of protein, starch, and calories as in the experiments with corn-meal. The other foods of the experimental diet were substantially as in the corn-meal experiments. Subject O here took the same amount of oat protein which she had taken of corn protein previously. Subject P took amounts of food approximately in proportion to her higher body weight. The main periods of the oatmeal experiment were made parallel to that in which Subject O had taken with the corn-meal diet 100 gm. of milk per day and had stored a small amount of nitrogen. With the oatmeal diet and 100 gm. of milk, Subject O showed nitrogen equilibrium and Subject P a slight storage. In the shorter periods without milk the losses of body nitrogen though small were slightly larger than in the corresponding experiments with corn-meal. It is doubtful if the differences are larger than may be due to the unavoidable fluctuations of nitrogen output in such experiments. The results indicate therefore that the protein of the oatmeal was practically as efficient as the protein of wheat flour or corn-meal.

With all three of the cereal grains tested (wheat, maize, and oats) it was found that a diet, in which about nine-tenths of the protein was derived from the cereal in the form commonly used as human food, the small remainder being furnished by milk or

apple, need contain only 33 to 40 gm. of protein per 70 kilos of body weight, or about 0.5 gm. per kilo, in order to meet the protein requirement of maintenance in adult human nutrition.

The proteins of wheat, corn, and oats appear to be about equally efficient in adult human nutrition and need only be supplemented with small amounts of milk in order to be fully as efficient as the proteins of ordinary mixed diets have been found to be in earlier investigations. Our findings for these cereal proteins are therefore similar to that of Hindhede for wheat bread and show their efficiency to be much higher than was reported by Karl Thomas for either wheat or maize.

Thus from the more recent and more carefully controlled experiments it appears that, even when the protein of the food is almost entirely derived from bread or other grain products, with a diet adequate in energy value a daily intake of about 0.5 gm. of protein per kilo of body weight is sufficient to meet the actual requirements of maintenance in healthy men and women. While if numerous older experiments having a tendency to high results are included the average is somewhat less than $\frac{2}{3}$ gm. of protein per kilo of body weight. A standard allowance of 1 gm. of protein per kilo of body weight per day appears, therefore, to provide a margin of safety of 50 to 100 per cent as far as the requirements of adult maintenance are concerned.

It is plainly desirable in all cases that grain products be supplemented by milk products, and it is clear that in providing for needs of growing children and of pregnant or nursing mothers the proportion of milk in the diet should be more liberal than it need be when only maintenance is concerned; this both because of the superior amino-acid make-up of the milk proteins and to provide amply for the mineral elements and vitamins as well.

EFFECT OF IODIDES ON THE AUTOLYSIS OF LIVER TISSUE.

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INTRODUCTION.

The medical literature affords much information concerning the therapeutic value of iodides among which may be mentioned germicidal action, stimulation of the production of opsonins, causation of leucocytosis, increased flow of lymph, and promotion of absorption by increasing the permeability of the vessel walls, but very little can be gathered regarding the chemical changes which the various iodine compounds undergo in the body. Their action on the tissue of the liver, lung, and spleen has been studied by Kepinow (1), but faulty technique has in general yielded inconclusive results as Kaschiwabara (2) demonstrated. Administration of KI was found to accelerate autolysis by Stookey (3) and Kepinow. KI added directly to comminuted liver tissue gave no or little acceleration. The slight acceleration is attributed by Morse (4) to increased acidity from liberation of HI. It is difficult to see how this could occur and no experimental proof is furnished. Considering the question of autolysis from the standpoint of protein cleavage, the literature offers but little information. I have therefore undertaken to examine the effects of NaI on the autolysis of liver proteins.

EXPERIMENTAL.

In order to make the results of the investigation comparable with those of others, the methods of Bradley and Morse (5) were employed with the introduction of a few minor modifications; first, instead of filtering the digest through filter paper it was

Dissolved amino nitrogen, control, <i>mg.</i>	1	7.20	6.40	5.60	4.60	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
	2	9.20	10.40	9.80	10.80	9.80	9.80	9.80	9.80	9.80	9.80	9.80	9.80
	4	12.20	13.00	12.20	12.00	11.20	11.20	11.20	11.20	11.20	11.20	11.20	11.20
	6	15.00	15.20	16.60	13.60	13.40	13.40	13.40	13.40	13.40	13.40	13.40	13.40
	8	15.80	16.20	17.00	15.80	16.60	16.60	16.60	16.60	16.60	16.60	16.60	16.60
Dissolved amino nitrogen + NaI solution, <i>mg.</i>	1	7.20	6.40	5.60	4.60	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
	2	8.60	10.80	10.80	10.60	9.40	10.20	10.00	10.20	10.40	9.80	9.20	9.20
	4	11.80	13.00	13.00	12.40	11.20	12.20	12.20	11.20	13.40	12.00	13.60	13.60
	6	14.60	15.80	16.20	14.00	15.60	15.40	14.60	14.60	14.20	13.40	13.60	13.60
	8	16.00	16.80	17.20	15.20	16.40	15.60	16.60	16.80	15.40	13.80	13.80	13.80
Cleavage nitrogen control, cc.	2	2.06	3.20	3.10	1.75	2.75	2.75	2.75	2.75	2.75	2.75	2.75	2.75
	4	4.20	4.16	4.66	2.35	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
	6	5.00	5.00	5.00	2.55	4.51	4.51	4.51	4.51	4.51	4.51	4.51	4.51
	8	5.36	5.30	5.36	3.15	5.21	5.21	5.21	5.21	5.21	5.21	5.21	5.21
Cleavage nitrogen + NaI solution, cc.	2	1.90	3.20	3.20	1.75	2.71	2.65	2.65	2.65	2.75	2.75	2.75	2.75
	4	4.20	4.20	4.60	2.35	3.81	3.95	3.81	3.81	3.91	3.85	3.81	3.81
	6	4.90	4.90	5.06	2.75	4.41	4.65	4.51	4.45	4.55	4.21	4.71	4.71
	8	5.36	5.40	5.40	3.01	5.25	5.35	5.15	5.15	5.21	4.61	5.25	5.25
Cleavage ammonia nitrogen, control, cc.	2	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
	4	0.40	0.40	0.40	0.50	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
	6	0.50	0.40	0.50	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
	8	0.70	0.50	0.50	0.70	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60

TABLE I.—*Concluded.*

NaI solution, per cent.	0.03	0.03	0.06	0.1	0.1+	0.25+	0.5+	0.75+	1.0+	1.5+	2.0+
	13.0	13.0	13.0	11.10	11.90	11.90	11.90	11.90	11.90	11.90	11.90
Total nitrogen, cc.											
	<i>days</i>										
Cleavage ammonia nitrogen + NaI solution, cc.	2	0.20	0.10	0.20	0.20	0.30	0.20	0.30	0.20	0.20	0.20
	4	0.40	0.40	0.30	0.40	0.50	0.60	0.70	0.60	0.60	0.60
	6	0.60	0.50	0.40	0.60	0.60	0.60	0.70	0.60	0.60	0.70
	8	0.70	0.60	0.60	0.80	0.70	0.60	0.80	0.60	0.60	0.70
Cleavage amino nitrogen, con- trol, mg.	2	1.80	2.00	3.60	2.80	3.80	3.80	3.80	3.80	3.80	3.80
	4	4.40	5.40	6.60	6.20	9.20	9.20	9.20	9.20	9.20	9.20
	6	7.80	7.20	10.20	8.80	10.60	10.60	10.60	10.60	10.60	10.60
	8	8.20	9.40	11.00	11.60	13.60	13.60	13.60	13.60	13.60	13.60
Cleavage amino nitrogen + NaI solution, mg.	2	2.00	2.80	3.00	3.40	4.00	4.20	4.20	3.60	3.20	4.20
	4	4.80	5.20	6.00	6.60	8.60	9.20	8.20	10.00	8.40	8.80
	6	8.00	7.80	9.60	8.60	10.80	9.80	11.00	10.60	10.60	11.20
	8	8.40	9.20	11.40	12.00	12.80	12.80	13.00	11.80	10.60	11.40

centrifuged until the supernatant liquid was free from visible solid particles, and second, the tannin precipitation method (6) was replaced by the aluminium cream method (7). Ammonia and amino-acid production during the process of liver autolysis in presence of iodides has never been determined.

DISCUSSION.

According to Morse, KI solution of a concentration of 0.2 to 1.0 per cent slightly accelerates autolysis, with inhibition above 1.0 per cent. The results of this investigation confirm his findings only to some extent.

The results in Table I are expressed in cc. of 0.2 N HCl, except that amino nitrogen is expressed in mg. per 1 gm. of fresh liver tissue. The figures in Column 2 indicate the intervals of days: 1 = immediately; 2 = after 2 days; 4 = after 4 days; 6 = after 6 days; 8 = after 8 days.

Dissolved nitrogen (total), dissolved ammonia nitrogen, and dissolved amino nitrogen were determined in the centrifugized digest.

Cleavage nitrogen (total), cleavage ammonia nitrogen, and cleavage amino nitrogen were determined in the filtrate from the aluminium cream precipitation method.

The results in Table II are obtained by subtracting the control figures from those of the iodides, and the result is multiplied by 100. In concentrations of sodium iodide from 0.1 to 1 per cent an increase is observed especially in the dissolved nitrogen, in the earlier days of incubation. Higher iodide concentration, from 1.5 to 2 per cent, tends also to give higher figures for this nitrogen. However, this proves by no means that NaI is responsible for this increase as far as its action as stimulus for proteolysis is concerned. On the contrary if sodium iodide behaves as other inorganic neutral salts do, according to Preti (8), it would inhibit instead of accelerate proteolysis, when employed in these concentrations. There is further a possibility that the ionized sodium iodide is responsible for the formation of soluble ion-protein compound which behaves in many respects as soluble protein.

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A slight variation in dissolved ammonia nitrogen and cleavage ammonia nitrogen is noticed, which I am not in a position to explain.

TABLE II.

NaI solution, per cent.	days	0.03	0.03	0.05	0.1	0.1	0.25	0.5	0.75	1.0	1.5	2.0
Dissolved ni- trogen (N), cc.	2	-36	-24	0	26	6	80	60	40	40	10	-4
	4	-10	-10	-46	30	10	6	30	10	20	0	16
	6	-10	-10	-26	-4	-16	-26	4	-6	-56	-50	4
	8	-14	-4	-4	16	-6	-20	34	-10	-26	-30	10
Dissolved am- monia nitro- gen (NH ₃ - N), cc.	2	0	0	-10	0	0	0	10	20	0	0	0
	4	0	-10	-20	10	10	0	20	20	20	10	20
	6	0	-10	0	10	0	0	0	0	0	0	10
	8	-10	0	0	10	10	0	10	0	0	0	10
Dissolved am- ino nitrogen (NH ₂ -N), mg.	2	-60	40	100	-20	-40	40	20	40	60	0	-60
	4	-40	0	80	40	0	100	100	0	220	80	240
	6	-40	60	-40	40	220	200	120	120	80	0	20
	8	20	60	20	-60	-20	-100	0	20	-120	-280	-280
Cleavage ni- trogen (N), cc.	2	-16	0	10	0	-4	-10	-10	-10	0	0	0
	4	0	4	-6	0	6	20	6	6	16	10	6
	6	-10	-10	6	20	-10	14	0	-6	4	-30	20
	8	0	10	4	-14	4	14	6	6	0	-60	4
Cleavage am- monia nitro- gen (NH ₃ - N), cc.	2	0	-10	0	0	0	10	0	10	0	0	0
	4	0	0	-10	-10	30	10	20	30	20	20	20
	6	10	10	-10	0	10	0	0	10	0	0	10
	8	0	10	10	10	10	10	0	20	0	0	10
Cleavage am- ino nitrogen (NH ₂ -N), mg.	2	20	80	-60	60	20	40	40	40	-20	-60	40
	4	40	-20	-60	40	-60	-60	0	-100	80	-80	-40
	6	20	60	-60	-20	20	120	-80	40	0	0	60
	8	20	-20	40	40	-140	-80	-80	-60	-180	-300	-220

The results of dissolved amino nitrogen are high compared with the cleavage amino nitrogen. This stands to reason, taking into consideration the methods employed. Aluminium cream will precipitate colloidal material, which will not be removed by the centrifugalization method, hence the figures of the latter are higher.

CONCLUSION.

It is evident from the results of this investigation that sodium iodide in concentrations of 0.03 to 2 per cent added to a suspension of liver pulp has no effect on the autolysis of this tissue.

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I. A CHEMICAL STUDY OF THE BLOOD OF SEVERAL INVERTEBRATE ANIMALS.*

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A study of the literature reveals the existence of comparatively few communications related to this particular field. The earliest work appears to be that of Macallum (1), dealing with the inorganic constituents of the blood of vertebrates and invertebrates. Other investigations somewhat closely related were made by Lipschütz (2), on the metabolism of fishes during starvation; Botazzi (3), on the peritoneal fluid and blood of sea animals; Jona (4), on freezing point values of the blood and body fluids of certain mammals, fish, and crustacea; Okuda (5), dealing with the quantitative determination of creatinine and creatine in some fishes, mollusks, and crustacea; and Fandard and Ranc (6), on sugar in the blood of the sea turtle.

The results embodied in these communications are greatly limited, however. The earlier workers were handicapped by the lack of accurate methods, and hence could not extend their studies to any great length. In later years the only work which has a comparative value when studied with that of the present paper is that of Denis (7), and of Wilson and Adolph (8), dealing with the determination of certain nitrogenous substances in the blood of fresh and salt water fishes; *e.g.*, shark, ray, mackerel, carp, and a few others.

In view of the general lack of information concerning the composition of the blood of invertebrate animals, it is evident that any substantial addition to our knowledge in this field

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would be of distinct interest. The immediate object of this investigation was the collection of chemical data along this line, with the added purpose of making a comparative study of the data obtained with relation to that from the more highly evolved animals. It is hoped that this study may bring to light a certain number of general relationships, which will enlarge our ideas of general metabolism, and, more remotely perhaps, assist the zoologist in tracing out some of the less clear biogenetic relationships.

The following invertebrate forms were studied.

Celenterates: The jellyfish, *Phacellophora camtschatica*.

Echinoderms: Two species of starfishes, *Pisaster ochraceus* and *Picnopodia helianthoides*. One species of sea urchin, *Strongylocentrotus francescames*.

Mollusks: Two species of clams, *Schizotherus nuttalli* and *Saxidomus nuttalli*. Two species of abalone, *Haliotis rufescens*, and the supposedly primitive molluscan form *Cryptochiton stelleri*.

Crustaceans: Two species of crabs, *Cancer productus* and *Cancer antennarius*. In this case the blood of the two species was mixed.

All the samples of blood were collected from the living animal, (a) by exposing the deeper surfaces, (b) by severing wholly or in part a blood vessel, and (c) by maceration of the tissues.

Where it was possible a specific gravity determination was made, and this was followed by a quantitative and qualitative chemical examination. The quantitative determinations made were total nitrogen, non-protein nitrogen, urea and ammonia nitrogen, amino-acid nitrogen, preformed and total creatinine, uric acid, sugar, cholesterol, chlorides as sodium chloride, calcium as calcium oxide, total solids, and ash. The qualitative tests included the reaction to litmus, the biuret, Millon's, Hopkins-Cole, xanthoproteic tests, and that for loosely combined sulfur.

In general the chemical examination was made directly after the blood was collected. Oxalate or citrate was added only in those cases in which experience had shown that coagulation would occur before the analysis could be undertaken. The samples were kept in tightly stoppered containers which were placed on ice. Toluene was added when conditions made this step advisable. Quantitative determinations were usually run in dupli-

cate, sometimes in triplicate. Analyses were repeated in all cases where there was the least doubt of the accuracy of the results. Relatively complete analyses were made, when the quantity of blood in a given sample permitted this to be done. In several cases repeated analyses of the blood from the same species of animal, collected at different times, were conducted. Whole blood was used in every case, and was composite, from two or nineteen or twenty forms of the same species being represented in the samples analyzed.

The methods followed in the collection of the samples were different for the various species studied. While the jellyfish, *Phacellophora*, possesses no celomic fluid or blood, it was included on account of its zoological position. Consisting largely of amorphous jelly-like mesoglea, quite firm to the touch, it was readily reduced to a fairly homogeneous liquid by gentle maceration through cheese-cloth.

In the case of the starfish, as much as possible of the extraneous sea water was removed, and then several of the rays were clipped off a few centimeters from their distal ends. The exuding liquid was allowed to drain into vessels arranged for the purpose. In the later samples the liquid was filtered through a loose tuft of absorbent cotton.

The procedure was slightly different for the sea urchin. In this case several incisions were made in the membrane surrounding the oral cavity. The animal was drained in the manner previously described, and the liquid filtered as before.

On account of the rather definite circulatory system of the mollusks, the procedure used in the preliminary stages of the work was limited to the selection of some of the larger blood vessels. Later on, however, a quicker and easier method was devised. Rather deep cavities were cut in the foot, for example, of the abalone, *Haliotis rufescens*, or in the gilt-cleft of the *Cryptochiton stelleri*. The blood filled these cavities rapidly and was removed by means of a small pipette with a curved end connected with a suction flask. Or in the case of the abalone, a part of the shell was broken and one of the branchial vessels exposed. This was partially severed, a fairly large hypodermic needle inserted, held securely in place with a small pair of forceps, and the blood carried over into a flask by suction. For

some unknown reason this method did not always yield an abundant supply of blood. A similar method was used for clams. The shell was broken and the position of the heart determined. The pericardial membrane was then severed as well as the superior vena cava and the blood running into the cavity removed in the manner previously described. This, however, is a tedious process. In order to facilitate matters the exposed body of the clam was superficially cut in several places and then gently macerated. The liquid collected was filtered through loose cotton. Total nitrogen determinations made showed a value of 39.98 mg. per 100 cc. of blood obtained in this manner, as compared with 39.92 mg. in blood collected from the pericardial cavity. Thus the difference is slight and the maceration method is to be preferred for simplicity as well as quickness.

The methods used for collecting crab blood were not essentially different from those previously described. Coagulation of the blood of these animals proceeds very rapidly after it is drawn—fibrin frequently separating out before sufficient oxalate is dissolved to prevent it.

Methods of Analysis.

Methods for blood analysis have thus far had a rather narrow application. The protein nitrogen for vertebrate blood has varied in round numbers from 2,800 to 3,700 mg. per 100 cc. of blood. The limits of the mineral constituents perhaps could be truthfully set at 400 to 600 mg. per 100 cc. These values are in decided contrast to those obtained in this investigation from the blood of invertebrate animals. Here the limits for the protein nitrogen vary from 1.5 mg. in the twenty ray starfish to 358 mg. in the crab, and for the mineral constituents from approximately 2,500 to 3,000 mg. per 100 cc. in the various species studied. In fact, comparatively speaking, these numbers are just the reverse in magnitude of those for the common vertebrates. In view of these striking differences, the presumption appears to favor the conclusion that occasional changes in the ordinary analytical procedures would be necessary. Experience in general has justified this view.

For preliminary removal of proteins the 50 per cent solution of trichloroacetic acid, used by Greenwald (9) and others, was by far the most satisfactory precipitant examined.¹ In some instances one precipitation was all that was necessary, the filtrate remaining free from any insoluble matter after considerable concentration. In many cases, however, it was necessary to digest the mixture in boiling water for 15 or 20 minutes to obtain the best effects. Enough of the acid was used to produce a concentration of from 6 to 8 per cent.

The freshly prepared 25 per cent solution of *m*-phosphoric acid recommended by Folin and Denis was not so satisfactory. The filtrates were in general cloudy, and even digestion in boiling water, a procedure which might be questioned, failed to accomplish the end desired. Concentration seemed to be the only recourse. There is, of course, the possibility always present of a union between the acid or some of its derivatives and the protein to form soluble products. This effect is very likely to take place when *m*-phosphoric acid is used, according to Folin and Denis, particularly where the acid is not fresh. Heating would of course favor this condition. Whether trichloroacetic acid would react similarly does not seem to be known, but experience seems to be against such a possibility. In any case any solution of the protein would vitiate the whole procedure.

Picrate-picric acid solution proved a most excellent precipitant, but its special character limited its use.

Alcohol was also used, but evaporation always yielded appreciable quantities of protein.

0.01 *N* acetic acid as a general precipitant was to be criticized in much the same way as *m*-phosphoric acid.

Kaolin as an absorbent of protein was satisfactory.

For total nitrogen, the Kjeldahl-Gunning method was followed, a small quantity of copper sulfate serving as catalyzer.

Non-protein nitrogen was determined by the method of Folin and Denis (10) and also by applying the Kjeldahl method to the protein-free filtrate. On account of the difficulty attendant on the absorption of

¹ The recently described protein precipitant for use in blood analysis, tungstic acid, recommended by Folin and Wu (Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81), was not used, since the investigation was nearly completed when the method came to the notice of the author.

color by the silicious insoluble matter, most of the determinations were made by the latter method. In most cases a sufficient supply of blood permitted this choice.

The determination of urea nitrogen was made by the methods of Folin and Denis (11) and Van Slyke and Cullen (12). Latterly the first of these methods was preferred, but with certain modifications. Instead of an emulsion of soy bean, a urease preparation was used either in the form of a powder or of a phosphate solution, according to Van Slyke and Cullen. The blood with the urease was digested at 45–50°C. for 30 minutes or longer, the remaining procedure being similar to that of Folin and Denis, except that 10 cc. of the filtrate were aerated into a known volume of a 0.2 N sulfuric acid solution to which was added ammonium sulfate equivalent to 0.05 mg. of nitrogen per cc., explanation of which will be made later. To expel the ammonia, 15 cc. of saturated potassium carbonate solution were used. The absorbent was then Nesslerized in the usual manner, a 100 cc. volumetric flask being used. The color comparisons were made by a Duboscq colorimeter with the standard set at 20 mm. Control determinations were made by subjecting urea solutions of known concentration to the action of urease, and completing the determination in the same manner as the unknown.

The determination of ammonia nitrogen was made according to the method of Folin and Denis (13) though with considerable modification influenced to some extent by the results of Barnett (14). 10 cc. of the blood were mixed with 15 cc. of saturated potassium carbonate solution and the mixture was aerated into a definite volume of 0.2 N sulfuric acid solution containing 0.05 mg. of ammonium sulfate per cc. The absorbent solution was then Nesslerized according to the method of Folin and Denis, and a colorimetric determination made with the standard set at 20 mm. Since a definite quantity of ammonia nitrogen was used to start with in the absorbent, this was subtracted from the total. The remainder represented the ammonia nitrogen in the blood. As will be noted a similar procedure was used in the determination of urea. The advantages of aerating into a standard solution of ammonium sulfate made acid with sulfuric acid are easily seen in this determination. The accessory apparatus of polarimeter tubes and condenser is eliminated. The depth of shade of the Nessler solution can be varied to suit the eye. Finally Nesslerizations can be made in 100 cc. volumetric flasks and after a little practice the standard can be so adjusted to the unknown that the reading will vary not more than 4 or 5 mm. from 20. The same remarks may well apply for the determination of urea nitrogen. The gain in simplicity is considerable.

In general the author does not look on aeration procedures with any great degree of favor. In spite of protective tubes and capacious aeration cylinders, it was necessary occasionally to make repeated determinations. The distillation procedure is perhaps more preferable.

The observation made by Barnett (14) that the quantity of ammonia nitrogen increases in the blood on standing was followed in its practical

significance by conducting the determination as soon as possible after the blood was drawn. That there is a decided increase in the quantity of ammonia on standing is in general agreement with the author's findings. This, it might be mentioned, is particularly true for whale blood.

Amino-acid nitrogen was determined by means of the Van Slyke (15) micro apparatus. In the initial treatment of the sample, the procedure of Bock (16) was followed. Several determinations were made, but only two are given in the table. In sea urchin, abalone, and crab blood values varying from 30 to 70 mg. per 100 cc. were obtained. The determinations were not always made directly after the blood was drawn. The post-mortem formation of amino compounds, or the action of certain substances in the blood on the nitrite used, might account for these high values. In any case there appear to be certain disturbing conditions.

Preformed and total creatinine were determined according to the method of Denis (17). In some cases 10 cc. of the standard were added to the unknown, and the determination was made. The picric acid used was tested according to the method of Folin and Doisy (18) and was found to satisfy the requirements. The principal difficulty experienced was the failure of color to develop after the prescribed amount of 10 per cent sodium hydroxide was added. Precipitates usually formed after the base was added. In order to overcome the first of these difficulties a cautious addition in excess of from 0.5 to 1 cc. of the base was made. Within narrow limits a variation in the base used had no influence on the reading when the experiment was conducted on standard creatinine solutions of the same concentrations. Precipitates were eliminated by centrifugalization. These difficulties appeared to be accentuated in determinations of total creatinine.

Uric acid was determined by the method of Folin and Denis (19). For amounts of uric acid which are found in human blood or of an approximate magnitude, no trouble was experienced. When only traces of the acid exist, and these in the presence of about 3 per cent sodium chloride, difficulties were encountered which were not entirely overcome. In order to render measurable the traces which appeared to be unmistakably present, from 30 to 50 cc. of the blood were used. The crystallizing out of considerable quantities of sodium chloride on evaporation appeared to vitiate the determination. Definite quantities of uric acid were then added to 3 per cent sodium chloride solution, but it seemed to be impossible to recover anything like the quantity of acid added. The large excess of sodium chloride may hinder perhaps the formation of silver urate by its mass action effect. Indications are that the method must be considerably modified to determine small quantities of uric acid under the conditions described.

Sugar was determined by the method of Lewis and Benedict (20). The picramic acid for the standard was prepared² according to the later method

² The work of preparation was kindly performed for the author by Dr. E. Oertly of Stanford University.

of Egerer (21). The only difficulty met with was in the application of the method to certain samples of blood such as that of the starfish, where the protein was very low. In this case the precipitate formed was difficult to remove by filtration. The precipitate appeared in the form of a suspension which did not readily settle.

For cholesterol, the method of Myers and Wardell (22) was used. Two modifications were introduced after several experiments. Instead of the standard "naphthol green B," a standard solution of Kahlbaum's cholesterol 1.0 mg. per 100 cc. in chloroform was used. The original procedure of taking an aliquot part of the extract was changed and the whole volume was concentrated to 5 cc. This modification was made necessary on account of the small quantities of cholesterol present, even when 3 to 5 cc. of the original blood were used.

It was found that the unknown as well as the standard cholesterol solutions possessed a decided bluish tint after performing the Liebermann-Burchard reaction. Hence good comparisons could not be made with the dye used as a standard. The blue shade observed is in decided disagreement with the observations made by Luden (23) in connection with the same reaction.

A final difficulty, not fully overcome, was the formation of a reddish shade in the extracts. This increased after adding the sulfuric acid and acetic anhydride. The shade seemed to develop to the greatest extent in clam blood. In the light of Luden's work the conclusion may be drawn that this color is due to icteric substances in the blood. At any rate, the color comparisons were made most difficult on account of this condition.

Chlorides as sodium chloride were determined according to the method of Rappleye (24). The method is simple and the end-point sharp for the chlorides in most invertebrate blood. When the sodium chloride is less than 0.6 per cent, the end-point becomes somewhat uncertain. Only 1 cc. of blood was used for the determinations, the amount of the silver nitrate solution varying from 15 to 20 cc. These quantities are slightly different from those prescribed by the method. In the author's view the method is considerably more simple than that of Van Slyke and McLean, and equally as accurate.

For total solids, ash, and calcium as the oxide, 5 to 10 cc. of the blood were evaporated to dryness in a weighed porcelain crucible over a water bath, and the residue was dried to constant weight at 110–115°C. This represented the total solids. The residue was then ignited, the temperature being kept as low as possible to avoid volatilization of the alkalies—a condition not always easy to accomplish in removing the last trace of the carbon—and then weighed. This represented the ash. The latter was then extracted with 15 to 20 cc. of concentrated hydrochloric acid, and the calcium as the oxide determined in general according to the method of McCrudden (25).

Specific gravity determinations were made at 20°C. by a pycnometer bottle.

The values in Table I exhibit several differences when compared with similar determinations made on the more highly developed animals. The exceptionally high content of inorganic salts, 2,900 to 3,200 mg., and the low protein nitrogen content, 0.7 to 340 mg. per 100 cc. of the blood, may be taken in illustration.

The influence of high saline content on osmotic pressure is obvious. The relatively small amount of urea and other organic crystalloids in the samples of blood examined clearly proves that the osmotic pressure is for the most part caused by the inorganic constituents. In any case the osmotic pressure is nearly the same as sea water ($\Delta = 1.81-2.8^\circ$) (26). In the more highly developed elasmobranch fishes this pressure is undoubtedly produced to a notable degree by the very large quantities of urea present in the blood, a constituent present in only small quantities in invertebrate blood. Neither the invertebrates examined nor the elasmobranch fishes appear to be independent of the surrounding medium in this regard. Neither set of forms has any means which enable them to influence their own osmotic pressure. This is in direct contrast to the marine and fresh water teleost fishes where probably a reduced saline as well as a urea content effects a material lowering of the osmotic pressure of $\Delta = 0.7^\circ$. As pointed out by Wilson and Adolph (27), these species are partially independent of changes in the surrounding medium. Undoubtedly the gills of these fishes have been modified in some way which enables them to adapt themselves to conditions of this kind.

That the low protein content of the blood of the invertebrate animals has a decided influence on its viscosity can hardly be doubted. Compared with the viscosity of the more highly developed marine forms and the mammals it should be considerably lower. In mollusk and crustacean blood the increased protein content would be parallel to an increased viscosity, and this in turn is associated with an improved cardiac and circulatory apparatus.

The buffer effects of the protein in mammalian blood in maintaining the required hydrogen ion concentration probably prevail in the blood of invertebrates, this value approximately being from 6.4 to 6.7 pH if the alkaline reaction to litmus is considered (Table II).

TABLE I.
Quantitative Data per 100 Cc. of Blood.*

Animal.	Specific gravity.	Solids.	Ash.	Ca as CaO.	Cl as NaCl.	Total N.	Non-protein N.	Urea + NH ₃ N.	Urea.	NH ₃ N.	NH ₃ amino N.	Creatinine.			Uric acid.	Sugar.	Cholesterol.	Remarks.
												Preformed.	Total.	mg.				
Jellyfish, <i>Phacelophora camtschatica</i> .						44	12	5.0				0.01	0.30	0.29		109		Obtained from Monterey Bay, Cal.
5 ray starfish, <i>Pisaster ochraceus</i> .				53	3,225	5	4.4	1.0	0	0		0.01	0.13	0.12			0.9	Obtained from Monterey Bay, Cal.
20 ray starfish, <i>Picnopus helianthoides</i> .				56	3,225	6	4.0	2.0								29	1.0	Obtained from Monterey Bay, Cal.
Sea urchin, <i>Strongylocentrotus francescames</i> .	1.024	3,485	2,992	63	3,140	12	8.6	1.00	0.92	0.08		0.11	0.27	0.16	Trace.		614.0	Obtained from Monterey Bay, Cal.
Clam, <i>Saxidomus nuttalli</i> .		4,330	2,800	307		44	11.0					0.28				50		Obtained from Moss Landing, Cal.
						59	13.0	10.0	0.95	0.95		0.31	0.74	0.43		77		
<i>Schizothorus nuttalli</i> .	1.029	4,208	3,290	193	3,190	40	14.0	3.6	2.94	0.66	8.0	0.38	0.80	0.42	Trace.		742.0	Obtained from Moss Landing, Cal.

TABLE II.
Qualitative Data on Samples of Whole Blood.

Animal.	Color of blood.	Reaction.				
		Litmus.	Xanthoproteic.	Millon.	Hopkins-Cole.	Lead.
Jellyfish, <i>Phacellophora cam-</i> <i>ischatica</i>	Colorless, macerate.	Slightly alkaline.	Medium.	Medium.	Quite strong.	Weak.
<i>Cryptochiton stelleri</i>	Dark am- ber.	"	Medium.	Medium.	Weak.	Weak.
Sea urchin, <i>Strongylocentrotus</i> <i>francescames</i>	Orange.	"	Quite strong.	Quite strong.	Strong.	Very strong.
Abalone, <i>Haliotis rufescens</i>	Light in- digo blue.	"	Medium.	Medium.	Weak.	Quite strong.
Clam, <i>Schizothorus nutalli</i> .	Bluish tint.	"	Strong.	Quite strong.	Very strong.	Strong.
Crab, <i>Cancer productus</i> and <i>antennarius</i>	Light greenish blue.	"				

The high values for calcium oxide, from 53 to 307 mg. per 100 cc. of blood are in accord with what one might expect in view of the considerable demand on the part of the invertebrate animals for the calcareous matter needed for the shell. The largest amount, 307 mg. per 100 cc. of blood, occurs in the *Saxidomus* clam. The shell of this species is much heavier than that of the associated *Schizothorus* clam. But if this criterion is taken to account for the difference in the amount of the calcium salts observed, it is somewhat difficult to account for the lower values 74 to 87 mg. per 100 cc. in abalone blood. A qualitative observation favors the view, however, that the proportion of the body of the clam to its shell is considerably less than in the case of the abalone.

Compared to several mammals, *e.g.* human blood 9.5 to 11 mg. per 100 cc., the calcium salts found in invertebrate blood may be several times as great.

The proportion of urea nitrogen to non-protein nitrogen appears to vary within rather wide limits. This is true for the echinoderms, 22 to 62 per cent of the non-protein nitrogen being represented as urea nitrogen in the blood of starfishes and 11 per cent in the one sample of sea urchin blood examined. In the mollusks and the one sample of crustacean blood the amounts vary from 7 to 70 per cent, though the mean value would fall more nearly between 10 and 20 per cent. The tendency then seems to be toward conditions similar to those which prevail in the blood of the teleost and ganoid fishes. The additional fact that the urea nitrogen is 40 per cent of the non-protein nitrogen in the flesh of the jellyfish examined has further interest.

The partition of the urea nitrogen between the form elements and the plasma was not studied. The form elements, however, comprise only a small proportion of the total nitrogen of the blood, it is believed. This appeared to be true particularly in the case of *Schizothorus* clam blood. The indications are that the urea is found principally in the plasma.

The ammonia, preformed creatinine, and total creatinine content of the blood of the echinoderms, mollusks, and crustaceans examined was in general considerably lower than the same constituents observed in the elasmobranch, teleost, and ganoid fishes (28). The amount of ammonia seemed to approximate more

closely that found in mammalian blood, though the creatinine and creatine are still lower even in this case. In addition to this the amount of creatine found in invertebrate blood was only a little greater frequently than the preformed creatinine. In starfish blood the creatine appears to be greatly in excess of the preformed creatinine, however.

The occurrence, at most only in minute traces, of uric acid in the blood of most of these animals agrees with the findings of Denis, in respect to the blood of the elasmobranch fishes. In the blood of the crab the amount of uric acid is relatively high, 4.7 mg. per 100 cc., and considerably in excess of that found in human blood; *e.g.*, 0.7 to 3.7 mg. per 100 cc. In this regard the metabolism of the crab appears to be similar to that of the birds and reptiles.

The amino-acid nitrogen probably makes up a large part of the remainder of the non-protein nitrogen, though only two determinations can be given to support this statement.

Sugar was found in all the samples of invertebrate blood examined, and varies from 29 to 90 mg. per 100 cc. of the blood. These values are similar in magnitude to those of human blood, *e.g.* 20 to 150, and perhaps other mammals. The only other determinations of sugar made on invertebrates were those of Fandard and Ranc (29) in the blood of a fasting turtle. Their values range from 82 to 95 mg. per 100 cc. of the blood, which are in agreement with those of the author for abalone and crab blood. The extreme variation does not appear to be wide in any of the animals examined. In respect to the invertebrate blood, it is interesting to speculate on the manner in which these animals maintain the amounts of sugar observed, in the presence of so much sodium chloride. At least in man the injection of 1 per cent salt solution intravenously becomes a glycuretic in causing a decided increase of the blood sugar in the urine. The relatively large amounts of calcium salts in the blood of invertebrates may lessen the permeability of the excretory membranes as they do apparently in man (30).

The quantity of cholesterol found was small in all the bloods examined, the variation extending from about 1 to 6 mg. per 100 cc. of blood. Compared with the values given for human blood (30 to 60 mg. per 100 cc.) they appear to be very low.

There seems to be considerable scarcity of data for cholesterol in the blood of mammals as well as of fishes. Hence any comparison must be extremely limited.

Finally it is of extreme interest to note the occurrence of several of the combined amino-acids, *e.g.* tyrosine, tryptophane, and cystine, in the tissue and the blood of these marine forms (Table II). If the qualitative tests are significant, the relatively large amounts of combined cystine and tryptophane in abalone and crab blood are most interesting.

SUMMARY.

The following determinations were made on the blood of several invertebrate animals: Specific gravity, total solids, calcium as calcium oxide, chlorine as sodium chloride, total nitrogen, non-protein, urea, ammonia, and amino-acid nitrogen, preformed and total creatinine, uric acid, sugar, and cholesterol. These values are found in Table I.

The osmotic pressure of the blood of these animals is about the same as sea water, and in this respect they agree with the elasmobranch fishes; *e.g.*, shark and ray. The major part of the osmotic pressure is due to the high saline content and not to the urea as in the elasmobranch fishes.

The very possible influence of low protein content in the blood on the lowered viscosity and the probable increase of this factor in the blood of the Mollusca and Crustacea is accompanied by a more highly developed circulatory system.

The urea content is relatively low, though the mean value is higher than that observed in some of the marine and fresh water fishes.

The ammonia, preformed creatinine, and creatine were generally lower than in the blood of the vertebrate fishes and other mammals.

The amino-acid nitrogen probably makes up the larger part of the non-protein nitrogen.

Sugar was found in the blood of every invertebrate examined and in relatively large quantities. In comparison with the sugar in the blood of other forms, the differences were not great.

Cholesterol was found in very small quantities. In comparison with the blood of man, the amounts were low.

The combined amino-acids, tyrosine, cystine, and tryptophane, the two latter in considerable quantities, were shown to be present in most of the blood(s), and in one case the tissue, of the invertebrate animals examined.

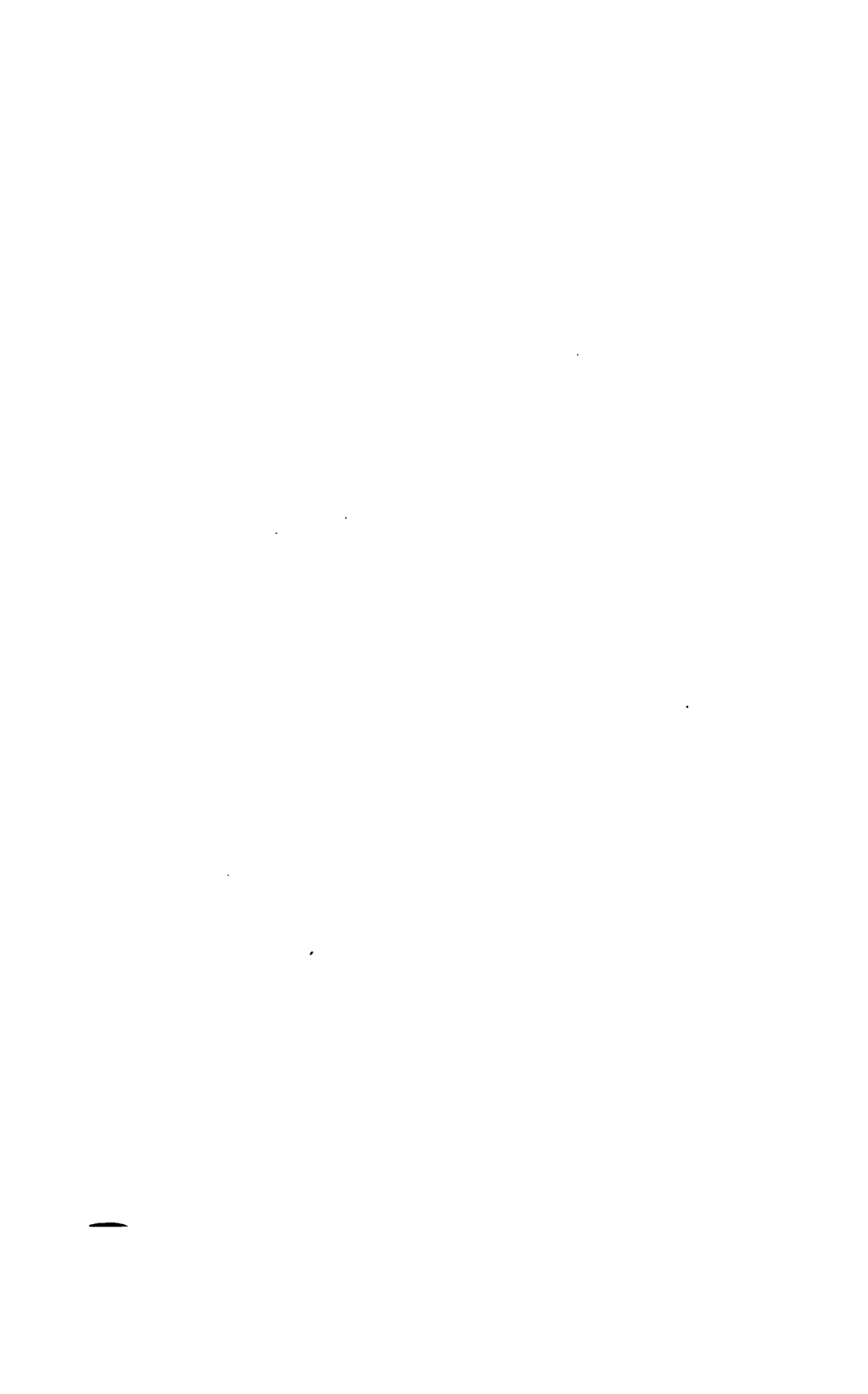
In conclusion the author wishes to express his great indebtedness to Professor R. E. Swain, whose many suggestions and practical assistance had a most happy influence on the success of this investigation as well as that connected with whale blood reported in the following paper.

In connection with the collection of the blood samples, the author wishes to acknowledge also the invaluable assistance of Professor Walter K. Fisher of the Hopkins Marine Station of Stanford University, Monterey, California.

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II. A CHEMICAL STUDY OF WHALE BLOOD.*

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Stanford University, Palo Alto.)

(Received for publication, November 17, 1919.)

A study of the literature revealed an almost complete lack of any work done in connection with the chemistry of whale blood. There seems, indeed, to be a general lack of information in respect to the zoological side as well. This paper then becomes an entirely new addition to the chemical knowledge of the whale, in particular to that of its blood. While not extensive, as far as repeated analyses are concerned, the work at least is a beginning for more extended examinations.

Two species only are included; the humpback whale *Megaptera versabilis* Cope, and the sperm-whale *Physeter macrocephalus* Linneus.

It was impossible to obtain the blood directly after the animal was killed. 3 or 4 hours, sometimes more, frequently elapsed before the sample was obtained. The blood was obtained from the freshly exposed and deeper muscular regions of the animal. The blood gushed from these regions in large quantities and no difficulty was experienced in getting it. It was quite warm and clotting ensued only some time after it was drawn. The ordinary methods failed to recover any fibrin from the blood of the humpback whale. The amount obtained from sperm-whale blood was relatively small compared to the blood used. Oxalate was added, except in samples where total solid determinations were made. The first humpback whale sample was taken from the thoracic cavity. The blood is probably not very pure, but it was decided to include it in the tabulation.

* This paper is part of a thesis presented to the Department of Chemistry of Stanford University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

TABLE I.

Animal.	Date.	Specific gravity.	Total solids.	Ca as CaO.	Cl as NaCl.	Total N.	Non-protein N.	Urea + NH ₃ N.	Urea.	NH ₃ N.	NH ₄ -acid N.	Creatinine.	Uric acid.	Sugar.	Cholesterol.	Remarks.
	1919		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
												Preformed.	Total.			
Humpback whale, <i>Megaptera versabilis</i> Cope. 1*	Mar. 24					1,177	325	160	156	4.0		2.2		400		
" 2†	" 11	1.038		15.8	580	2,237	221	137	122.5	14.5	59	17.6	39.2	21.6	42.6	Killed in Monterey, Bay, Cal.
Sperm-whale, <i>Physeter macrocephalus</i> Linneus.‡	May 1	1.061	22,025	13.7	509	3,518	116	74	71.6	2.4	31	5.3	14.2	8.9	2.1	Killed in the Pacific Ocean about 10 miles off Monterey Bay, Cal. The amount of protein by the Thierfelder method was 20,775 mg., the protein ash 195 mg., and soaps and lipins including cholesterol approximately 401 mg. per 100 cc.

* The blood was probably impure, since it was selected from drainings into the thoracic cavity.

† This animal was about 40 feet long and had an estimated weight of 35 tons. This species belongs to the whale bone whales. A larger species is the so called "sulfur bottom" or baline whale. The latter grow to the length of 95 feet. The humpback feeds on large quantities of sardines and anchovies. Their stomachs have been known to contain from 2 to 3 tons of these fish.

‡ The specimen from which the blood was obtained had a length of 65 feet 9 inches. The lower jaw was about 8 to 9 feet in length, and had 48 teeth 2 to 3 inches in diameter, and was narrow and tapering. The head was about one-third the length of the whole body. The weight of the animal was variously estimated at from 80 to 90 tons. Blubber was 6 to 8 inches in thickness. Of the spermaceti recovered, there were 90 barrels from the whole animal, 32 barrels coming from the head alone. The exposed intestines had a dark, though bright fluorescent green color. The stomach contained a shark 10 feet 2 inches long, the pigmented layer of whose skin only having been attacked by the gastric juices, the skin of a partially digested seal, and 8 feet of fishing line with six hooks. While the flesh of the humpback whale is used for food, the flesh of this species is not. The sperm-whale appears to be entirely carnivorous in habits, squid and devil fish being favored articles of diet. When wounded it will attack the pursuing boat, according to reports. The author was favored with most of the above data by Mr. Chester I. Dennis, an official of the California Sea Products Company, Moss Landing, Cal.

The methods of analysis employed were the same as those used in the examination of invertebrate blood (see page 122). Little if any difficulty was experienced in the application of these methods.

In contrast to invertebrate blood, *m*-phosphoric acid solution gave clear filtrates. These, however, precipitated small quantities of protein on evaporation.

In the cholesterol determinations the reddish shade noted in the previous paper was also present in the extracts of whale blood.

A study of Table I reveals noteworthy differences in the composition of humpback and sperm-whale blood. Not only are these differences exhibited in the protein content, and in the nitrogen partition, but in some of the other constituents as well. Our general ignorance of the habits and life of these animals renders somewhat abortive all attempts to account satisfactorily for these differences. Both animals appear to be carnivorous feeders, though the sperm-whale, in contrast with the humpback, devours larger animals. Many other conditions would have to be considered, however, and the whole question will remain obscure until our knowledge becomes more definite and extended.

In respect to the humpback whale the large quantity of urea in the non-protein fraction would have a considerable influence on the osmotic pressure of the blood. This would be considerably lower than that of sea water, of invertebrate and elasmobranch fish blood, though higher than that of the marine and fresh water teleost fishes. The value $\Delta = 0.8 - 0.9^\circ$, would perhaps be an approximation. The blood of the sperm-whale is considerably lower than this, the range of this value being similar in magnitude to that of the teleost fishes ($\Delta = 0.7^\circ$). The osmotic pressure of the blood of both species however is higher than that of the blood of several of the land mammals; *e.g.*, $\Delta = 0.6^\circ$. Evidently both species are relatively independent of changes in the medium, which would give them a wide foraging capacity. It is somewhat interesting in this connection to speculate on the ability of the whale to adapt itself to a fresh water medium.

The striking contrast in the amounts of protein in the blood of the humpback (13,981 mg.) and the sperm-whale (20,775 mg.)

per 100 cc.) suggests interesting possibilities. From one point of view the increased viscosity of sperm-whale blood might call for changes in the circulatory apparatus over that of the humpback whale. From another point of view the decreased buffer property of the protein in the latter species would lower its power over the former in absorbing acids or bases during metabolism. It is conceivable that this point could have considerable significance in relation to special conditions tending to disturb the hydrogen ion concentration of the blood, the sperm-whale being considerably favored in this regard over the other species. In respect to human blood, at least, the protein of the blood of the sperm-whale is practically the same in quantity. As can be seen this does not hold true for the humpback.

The percentage of urea nitrogen in the non-protein content is much the same for both animals. In sperm-whale blood it is slightly over 60 per cent and in humpback whale blood 48 per cent. These are high values for mammals. They are also in excess of those for the blood of invertebrates, as well as for the marine and fresh water teleost and ganoid fishes, the blood of only the elasmobranch fishes exhibiting a greater percentage.

The quantity of the amino-acid nitrogen is also greater than that found in land mammals (1). In addition to this it makes up the larger part of non-protein nitrogen after subtracting the urea nitrogen.

The creatinine and creatine content is also relatively high for mammals.

The amount of uric acid found was not so excessive though its range more closely approximates that present in birds and reptiles.

The amount of the inorganic constituents does not differ greatly from that observed in human blood and other mammals. The effect of these substances on the osmotic pressure is very slight in comparison with that in the blood of the invertebrate animals.

The total solids and the specific gravity appear to vary considerably from human blood (specific gravity 1.026 to 1.030; solids 21,000 to 24,000 mg. per 100 cc.). The specific gravity of sperm-whale blood is much higher, though the solids are of a mean value.

The large amount of sugar in humpback whale blood appears to be rather excessive compared with human blood; that of the

sperm-whale, however, is much the same as that found in the latter. In both species it is much higher than that observed in the blood of invertebrates.

The cholesterol values obtained in the blood of both species are within the range found in human blood (30 to 60 mg. per 100 cc.), though very high compared to the amounts found in the blood of invertebrates.

The composition of whale blood seems to show several anomalies to that of other animals. Doubtless the peculiar habits of this animal and the fact that it is a marine mammal may help to account for these differences.

Further it is interesting to speculate on the special character of a metabolism which not only elaborates larger quantities of fat but the wax, spermaceti (cetyl palmitate), as well. Such phenomena only serve to make more impressive the marvelous lability of the chemical processes involved in the general metabolism of animal life.

SUMMARY.

Analyses of the blood of the humpback and sperm-whale were made. These analyses include the determination of specific gravity, solids, calcium as calcium oxide, chlorine as sodium chloride, total nitrogen, non-protein, urea, and ammonia nitrogen, creatinine and creatine, amino-acid nitrogen, uric acid, sugar, and cholesterol. These data are to be found in Table I.

The osmotic pressure of the blood of both species is undoubtedly higher than in other mammals ($\Delta = 0.7-0.9^\circ$ approximately) but lower than that of the elasmobranch fishes and the invertebrates.

A considerable variation exists in the quantity of protein in the blood of the two species. In the sperm-whale it is nearly the same as that in human blood (21,000 mg. per 100 cc.). In the other species it is less than two-thirds as great.

The urea content is high for mammals, as well as the percentage of urea nitrogen in the non-protein fraction. Nor are the invertebrates or some fishes excepted in this respect, though the content of urea in the blood of the elasmobranchs exceeds that of whale blood.

The content of amino-acid nitrogen, of ammonia nitrogen, and of creatinine and creatine is generally higher in whale blood than in the other mammals, some marine invertebrates, and fish.

The uric acid content was not found to be excessive. It would probably range rather closely to that of birds and reptiles.

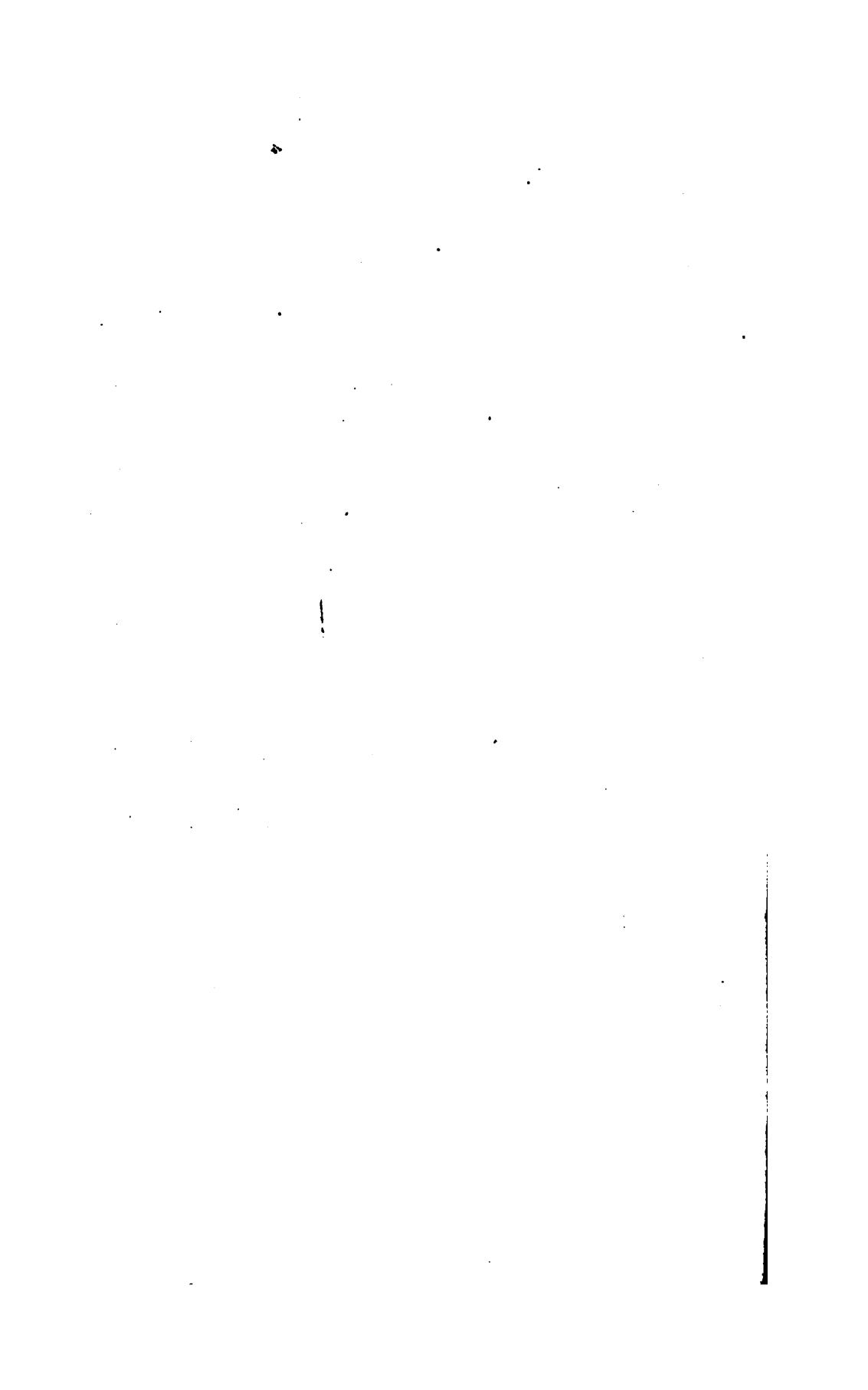
In respect to the amounts of the sugar and cholesterol, the former appeared to be excessive in the blood of only the hump-back whale, though in the blood of the other species the amount found was similar to that found in human blood. The amounts of cholesterol in the blood of both species had much the same range of magnitude as is observed in human blood.

The amount of the mineral constituents was much the same as in other mammalian blood.

The specific gravity of sperm-whale blood is much higher than that of human blood, and for the other species it closely approximates the latter.

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NOTE ON THE PREPARATION OF P-DIMETHYLAMINO-BENZALDEHYDE.

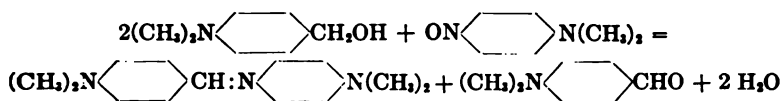
By T. INGVALDSEN AND L. BAUMAN.

(From the Department of Internal Medicine, State University of Iowa, Iowa City.)

(Received for publication, December 12, 1919.)

During the war there was a shortage of *p*-dimethylaminobenzaldehyde. This compound is the chief ingredient of Ehrlich's reagent which is used for the determination of urobilinogen in the urine, feces, and bile. As there appeared to be a demand for this substance by physicians and investigators it was desirable to attempt to simplify the method of preparation. Several methods are to be found in the literature. The method of Ullmann and Frey¹ appeared simple and easy of execution. It consists of the following steps: (a) The preparation of *p*-nitrosodimethylaniline; (b) the reaction of (a) with *p*-dimethylaminobenzyl alcohol; (c) the cleavage of anhydro-*p*-dimethylaminobenzaldehyde-*p*-aminodimethylaniline and liberation of the desired aldehyde.

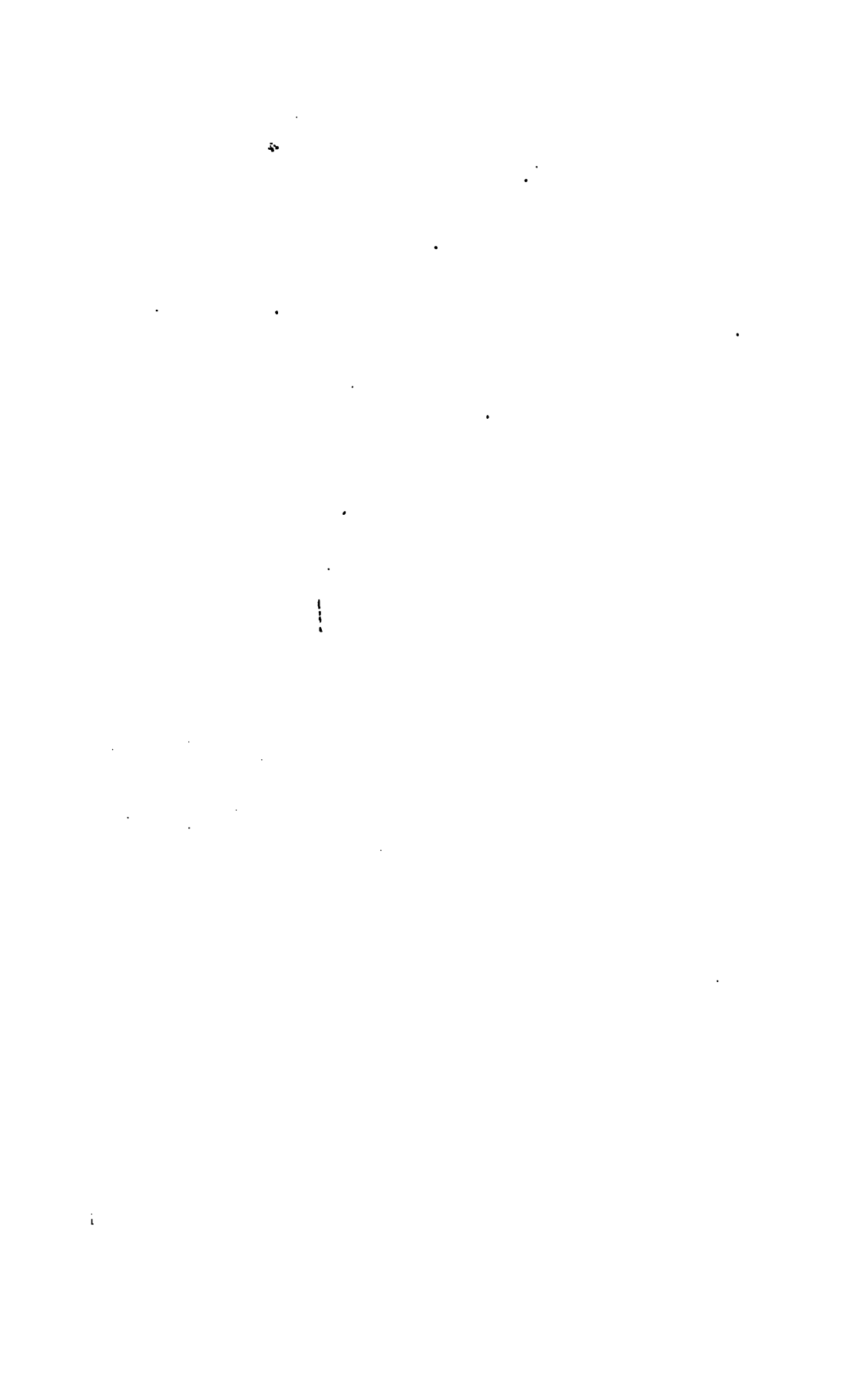
The reaction involved in step (b) is as follows.



As an excess of the nitroso body is used, the second molecule of aldehyde is partially converted into the benzylidene body.

Experience has led us to modify the procedure in several respects. The isolation of the hydrochloride of the benzylidene compound is omitted. The decomposition of the free base by formaldehyde and acetic acid is carried out at room temperature. The crude aldehyde is purified by distillation in a partial vacuum.

¹ Ullmann, F., and Frey, B., *Ber. chem. Ges.*, 1904; xxxvii, 855.



NOTE ON THE PREPARATION OF P-DIMETHYLAMINO-BENZALDEHYDE.

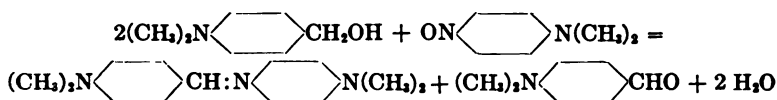
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¹ Ullmann, F., and Frey, B., *Ber. chem. Ges.*, 1904; xxxvii, 855.

Preparation of p-Dimethylaminobenzaldehyde.

300 gm. of technical dimethylaniline are dissolved in 1,500 cc. of dilute hydrochloric acid (1:1) and placed in a freezing mixture. A saturated solution of 180 gm. of sodium nitrite in water is added slowly from a dropping funnel while the reaction mixture is being agitated by a mechanical stirrer. The operation requires about 1 hour. The nitroso body is filtered with suction and washed with the dilute hydrochloric acid. In the meantime 360 gm. of dimethylaniline, 250 cc. of formaldehyde, and 600 cc. of concentrated hydrochloric acid are mixed in a large beaker which is heated on the boiling water bath for about 10 minutes after which the nitroso compound is added at once. The violent reaction which ensues is completed in about 5 minutes. After cooling and diluting with water, the base is precipitated by the addition of commercial sodium hydroxide (lye) until all the red color has disappeared. The solid is removed by filtration and washed with tap water.

The moist base is transferred to a heavy beaker and covered with 2,000 cc. of 50 per cent acetic acid and 500 cc. of commercial formaldehyde. The mixture is stirred until 20 minutes after the benzylidene compound has gone into solution. The aldehyde separates as a crystalline mass after adding 1,000 cc. of water and crushed ice. After 12 hours refrigeration the solid is filtered off and washed until the washings are perfectly clear. It is dried at room temperature. Yield 260 gm.

Purification is carried out by distillation of the *dry* aldehyde from an oil bath. At 43 mm. pressure it distills at 200°. The hot distillate is treated with 200 cc. of 95 per cent alcohol, poured into a large mortar, and stirred while distilled water is gradually added. All large lumps must be broken up with the pestle. The product is then transferred to a large beaker containing 1,000 cc. of water and 25 cc. of glacial acetic acid. Yield 250 gm., of pure product melting at 73°C. The approximate cost is about 5 cents per gm.

NOTE ON THE OXIDATION OF SUGARS BY MERCURIC ACETATE IN THE PRESENCE OF AMMONIA.

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(From the Department of Internal Medicine, State University of Iowa, Iowa City.)

(Received for publication, December 12, 1919.)

In the past a number of investigators have studied the oxidation of sugars by mercuric salts with the object of obtaining a method for their qualitative or quantitative determination. Herzfeld¹ found that glucose is readily oxidized by red mercuric oxide in the presence of barium hydroxide. Heffter² obtained gluconic acid by boiling a glucose solution with yellow mercuric oxide.

Ammonium gluconate and ammonium galactonate may be obtained in a 50 per cent yield by the action of mercuric acetate on glucose or galactose in the presence of ammonia. Mannose and lactose are also oxidized by this method but the ammonium salts of their respective acids cannot be isolated in crystalline form.

Preparation of Ammonium Gluconate and Galactonate.

10 gm. of glucose dissolved in 100 cc. of water are treated with 25 gm. of mercuric acetate and 15 cc. of concentrated ammonia solution and allowed to remain at room temperature over night. The solution is then placed on the water bath for 12 hours, saturated with hydrogen sulfide, filtered, purified with bone-black, and evaporated in a partial vacuum. Ammonium gluconate crystallizes when the remaining syrup is treated with alcohol. The crystals occur as thin hexagonal plates and melt at 155–157° (uncorrected). The physical constants are similar to those obtained by Irvine, Thomson, and Garrett³ and also to those of a

¹ Herzfeld, A., *Ann. Chem.*, 1888, ccxlv, 27.

² Heffter, A., *Ber. chem. Ges.*, 1889, xxii, 1049.

³ Irvine, J. C., Thomson, R. F., and Garrett, C. S., *J. Chem. Soc.*, 1913, ciii, 238.

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sample of ammonium gluconate obtained by the oxidation of glucose with bromine. The yield is 6 gm. from 10 gm. of glucose. The recrystallized substance analyzed as follows.

0.4156 gm. required 19.5 cc. of 0.1 N sulfuric acid (Kjeldahl).
Calculated for $C_6H_{12}O_7N$ 6.57 per cent. Found 6.56 per cent.
The specific rotation was $+13.63^\circ$.

$$[\alpha]_D^{20} = \frac{6.3192 \times +3.21^\circ}{1.3642 \times 1.0908}$$

Ammonium galactonate when prepared by the above method crystallizes in small needles. The yield is 7 gm. from 10 gm. of galactose. The melting point is $155-157^\circ$.

The recrystallized sample analyzed as follows.

0.3388 gm. required 15.9 cc. of 0.1 sulfuric acid (Kjeldahl).
Calculated for $C_6H_{12}O_7N$ 6.56 per cent of nitrogen. Found 6.57 per cent.

The specific rotation was $+3.33^\circ$.

$$[\alpha]_D^{20} = \frac{6.3116 \times +0.75^\circ}{1.2986 \times 1.0946}$$

FAT-SOLUBLE VITAMINE.*

IV. THE FAT-SOLUBLE VITAMINE CONTENT OF GREEN PLANT TISSUES TOGETHER WITH SOME OBSERVATIONS ON THEIR WATER-SOLUBLE VITAMINE CONTENT.

BY H. STEENBOCK AND E. G. GROSS.

WITH THE COOPERATION OF MARIANA T. SELL.

*(From the Laboratory of Agricultural Chemistry, University of Wisconsin,
Madison.)*

(Received for publication, December 6, 1919.)

While the mere isolation and the determination of properties of substances found in the plant and animal kingdom are most fascinating and stimulating procedures in the chemical laboratory, such work becomes especially engaging from the biochemical point of view, when speculation as to the rôle of the multitudinous array of compounds is injected into the work. Viewed from this angle, there is little wonder that the attention of many biochemists should, at devious times, have been absorbed in the work of isolating organic acids, carbohydrates, proteins, alcohols, esters, and bases with the prospect of securing an idea as to the origin, the interrelations, and the fate of these substances.

In animal physiology, work of this nature has yielded far reaching results in the formulation of our present day conceptions of the relations of the individual to his nutritional environment. Especially is this evident in problems pertaining to energy relations and those concerned with the constructive process of growth and maintenance. But, as these have been developed, it has become increasingly evident that, if our conception of physiological processes in various fields is to be built up symmetrically, it becomes imperative that information on the nature of the vitamins be accumulated so that their specific rôle in the animal body can be determined. Funk (1), and Braddon and Cooper (2), who sur-

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mised a relation between polyneuritis as caused by an insufficiency of the water-soluble vitamine and carbohydrate metabolism, and then Drummond (3), who investigated the effect of the water-soluble vitamine on nitrogen metabolism and the effect of the fat-soluble vitamine on fat metabolism, are the only investigators—as far as we know—who sought to associate the rôle of vitamines with the general body metabolism.

It is true that much of immediate practical importance in vitamine relations may be gained by a study of the dietary properties of various foodstuffs fed singly and in combinations, but it is not to be questioned that ultimately a true conception of problems in nutrition is dependent on the determination of the occurrence of various substances in foods and a development of an appreciation of their physiological rôle. We refer here to the effect of various substances on secretion, motor activity, irritability, conductivity, permeability, and cell proliferation all of which are concerned in such a gross physiological process as growth.

In the field of vitamine physiology, progress in the suggested direction is beset with many difficulties as shown by the fact that no vitamine has as yet been isolated. If it is not the lability of the compound in question, it is its extreme chemical indifference to such reagents as are ordinarily used to modify solubilities that prevents its separation from its environment. The present state of our knowledge is such that any indications as to the probable nature of a vitamine is worthy of investigation. In the case of the fat-soluble vitamine we have adopted as our working hypothesis (4) that it is either identical with or else chemically related to certain yellow plant pigments. With this as a clue, which is an outgrowth of their often observed association in nature (5), methods leading to the extraction of certain yellow pigments have given us many pigmented solutions carrying the fat-soluble vitamine. While this appears promising, it seemed imperative—before developing this work extensively—to accumulate information as to the best sources available for the isolation of this dietary essential and also to determine its stability in such materials.

Unfortunately data on the distribution of the fat-soluble vitamine are very limited in the literature, and, such as there are, were to a large extent obtained when the laboratory technique of determining dietary relations was not so highly developed as it is

at the present time. This situation has been discussed by Osborne and Mendel (6) in a preliminary communication in which they have submitted some data, but here again their data are not directly comparable with ours so that we have been obliged to continue with our accumulation of information to bring out what we shall present later. In the present paper we present data on the fat-soluble vitamine content of alfalfa, clover, spinach, lettuce, cabbage, and chard, and in addition present some data on their water-soluble vitamine content as well.

EXPERIMENTAL.

As in the previous papers of this series (5, 7, 8) the relative amount of fat-soluble vitamine present in the various plant tissues was determined by establishing the minimum amount—down to 5 per cent of the ration—necessary to satisfy the growth impulse of a recently weaned young rat for a period of at least 4 months. Using this technique it is, of course, necessary to have the young animals, as near as possible, of the same age and in good condition at the start of the experiment. Furthermore, it is imperative to know that in the experimental ration all other dietary requirements are complied with, so that growth is possible when a deficiency in the fat-soluble vitamine content does not occur or when it is corrected. Such a ration is readily formulated in a basal mixture consisting of purified casein, dextrin, agar, salts,¹ and water-soluble vitamine. The latter was usually incorporated as an alcoholic solution of ether-extracted wheat embryo, but sometimes as ether-extracted wheat embryo itself. As a rule, four experimental animals were fed in a group to eliminate individual differences by the law of averages.

Fat-Soluble Vitamine Content of Alfalfa.

Probably more of the statements relative to the fat-soluble vitamine content of leafy materials are based on data obtained with alfalfa than with any other materials, but unfortunately one is unable to glean from the information available, as pointed out

¹ For composition of salt mixtures see Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1919, xxxv, 517; Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.

by Osborne and Mendel (6), whether failure of growth, when it occurred, was due to lack of the fat-soluble or the water-soluble vitamine. These complications are avoided in the data presented in our Charts 1 to 5. The alfalfa used in these experiments was an early summer growth cut in full bloom when about 18 inches high. It was cured at room temperature in the diffuse light of the laboratory and was then ground to a meal.

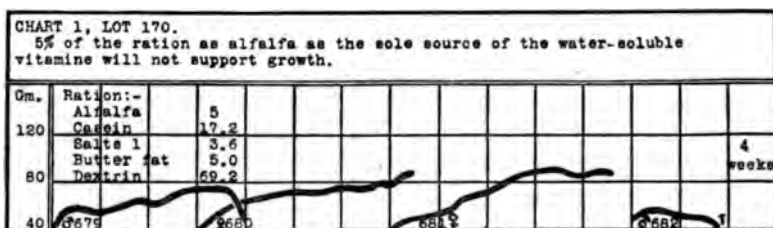


CHART 1.

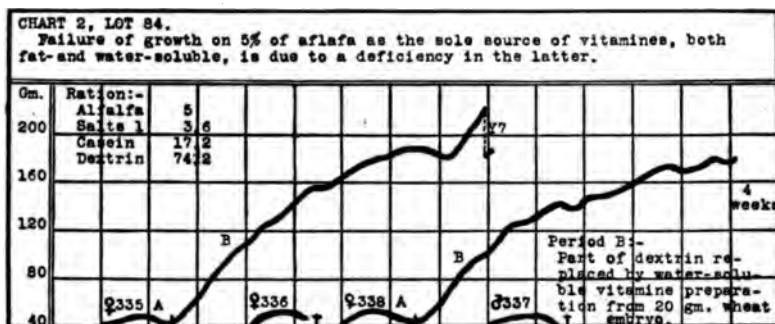


CHART 2.

Charts 1 and 2 demonstrate the deficiency of alfalfa in the water-soluble vitamine; in fact Rats 679 and 682 both went into convulsions as the result of polyneuritis. That the inhibiting factor really was the water-soluble vitamine is shown by the response in growth of Rats 335 and 338 when this dietary essential was added in an alcoholic extract of wheat germ.

In Chart 3 it is seen that growth is possible at the normal rate on a ration carrying 5 per cent of alfalfa as the source of vitamins if the water-soluble vitamine deficiency is corrected. Fur-

thermore, young were successfully reared. Rat 683 raised two young out of a litter of seven to an average weight of 42 gm. in 32 days. While the time for rearing was prolonged 8 to 10 days beyond the normal, the fact that the rearing of young was possible gives indisputable evidence of the richness of alfalfa in the fat-soluble vitamine as the other ingredients of the ration have repeatedly been shown to be free from it. How much less than 5 per cent of the ration might have been constituted of alfalfa and

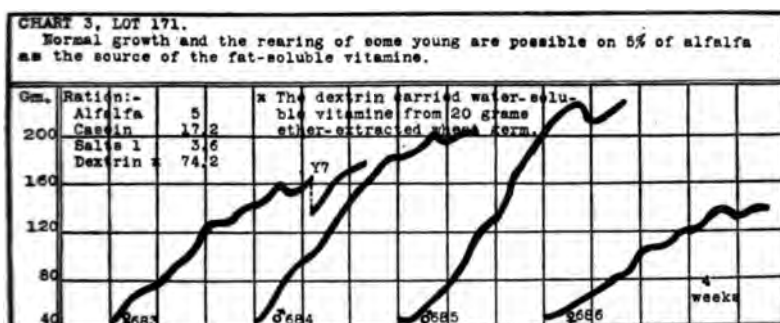


CHART 3.

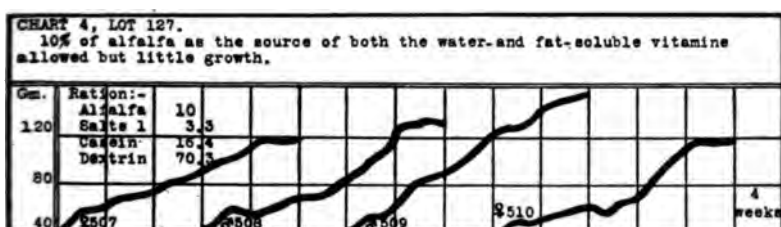


CHART 4.

still have produced results such as the above we have not determined, but we are inclined to think that with our material we had reached approximately the lowest level possible. This is suggested by the curves of growth and the behavior of the young.

Having established that normal growth and the rearing of some young are possible on 5 per cent of alfalfa no question of a fat-soluble vitamine deficiency could be raised in the case of a ration containing 10 per cent of alfalfa, as shown in Chart 4. Failure to grow at the normal rate must be attributed to a lack of a sufficient

amount of the water-soluble vitamine as in the lots pictured in Charts 1 and 2. No symptoms such as the convulsions of polyneuritis were observed nor were they expected as animals growing at the rate indicated will often continue their subnormal rate of growth for many months with no signs of collapse.

When the amount of alfalfa was increased to 15 per cent, Chart 5, the requirements of the rat for both the fat- and water-soluble vitamins were satisfied; normal growth and the rearing of young became possible. Rat 342 raised three young to an average weight of 43 gm. in 4 weeks.

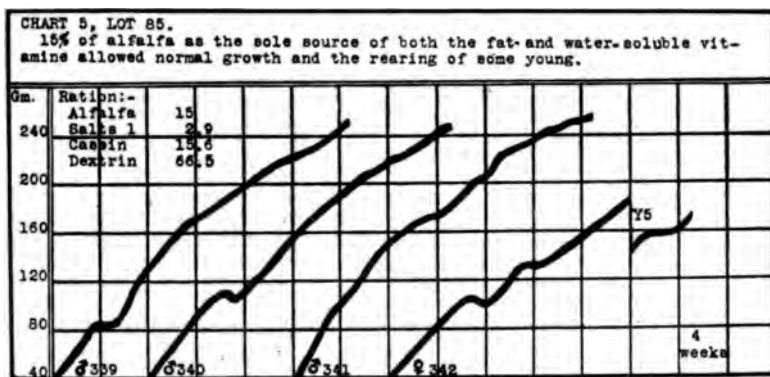


CHART 5.

Fat-Soluble Vitamine Content of Clover.

The clover used in these experiments was red clover which had completed its growth and almost its blossoming as only an occasional red blossom appeared among the numerous turning heads. It was dried in the laboratory at room temperature, then ground to a meal, and as such incorporated in the ration.

Chart 6 shows definitely that our clover fed at a 5 per cent level like the alfalfa (Chart 1) did not introduce sufficient water-soluble vitamine into our basal ration to allow growth. The fat-soluble vitamine on the other hand was introduced in considerable amounts with this amount of clover as is evident from the prompt inception of growth in Rats 2328, 2329, and 2331 when the water-soluble vitamine deficiency was corrected by the addition of an

alcoholic extract from ether-extracted wheat embryo. Rat 2330 had died as a result of lack of the water-soluble vitamins before the addition was made.

In Chart 7 are shown the curves of growth of rats on 5 per cent of clover as the source of fat-soluble vitamins in the ration when the water-soluble vitamin deficiency had been corrected from the

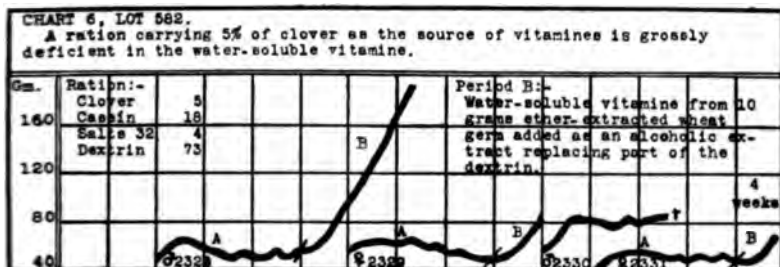


CHART 6.

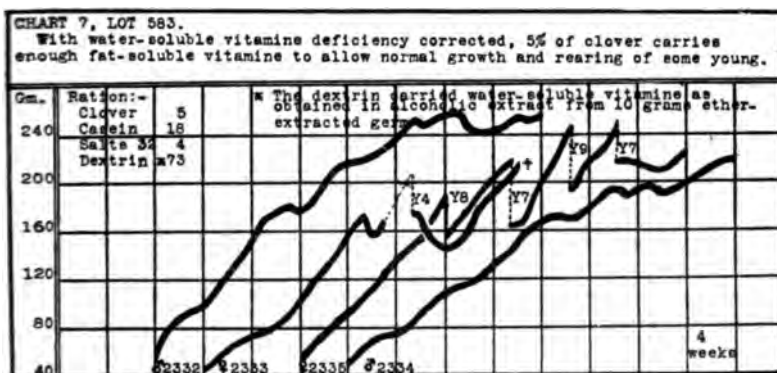


CHART 7.

very start of the experiment by an extract of wheat germ. Growth approximating normal was the result and, as in the case of alfalfa when fed at this level, young were reared though at a subnormal rate of growth. By way of illustration, Rat 2333 raised two out of a litter of four weighing 6 gm. as an average to an average weight of 36 gm. in 9 weeks. They were very small for their age as they should have had this weight when 3 weeks old, but they continued to grow on the ration and when discarded were rated as being

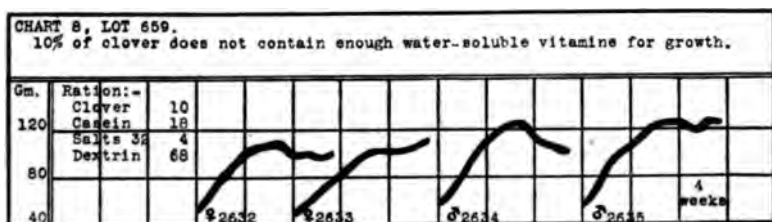


CHART 8.

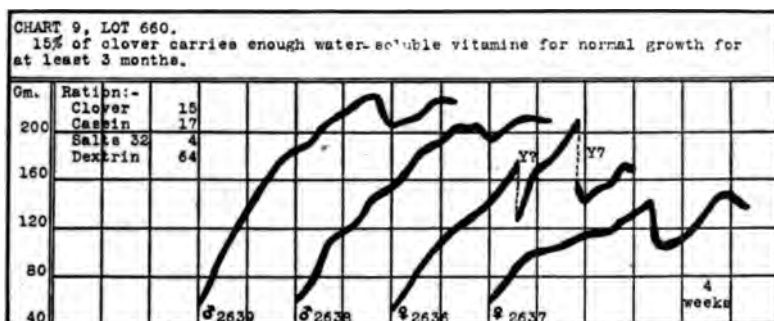


CHART 9.

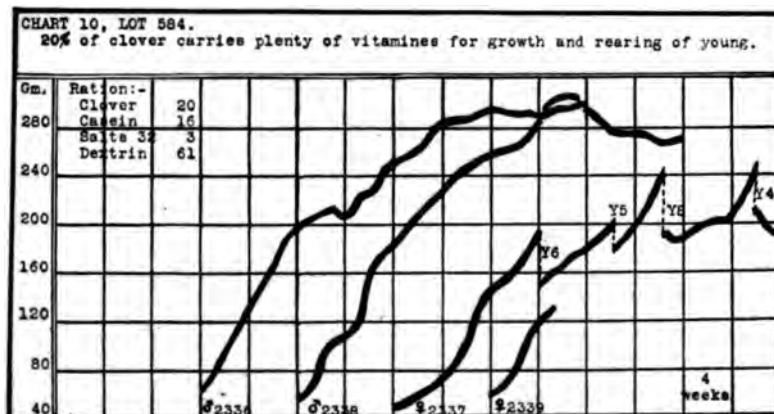


CHART 10.

in good condition. Rat 2335 did not raise her first three litters, but two out of her fourth litter were raised in 6 weeks and 4 days to an average weight of 42 gm., which again is distinctly sub-normal.

Having established the dietary efficiency of clover as a source of the fat-soluble vitamine when fed at a 5 per cent level, especially for growth, it must be concluded that the failure of growth when the clover was fed at a 10 per cent level as supplemented in our standard basal ration (Chart 8) must have been due to a water-soluble vitamine deficiency. When increased to 15 per cent (Chart 9) the rate of growth approached the normal. It was not, however, until 20 per cent of the ration was constituted of dried clover that growth was entirely satisfactory and that young were successfully reared (Chart 10). Rat 2339 was accidentally killed by the attendant, but Rat 2337 raised four of her litter of eight to an average weight of 44 gm. in 4 weeks. Her first and second litters were not raised which may, however, have been due to negligence as after parturition she was not segregated from the other members of this group. Under such conditions a perfectly healthy, well nourished animal sometimes discontinues nursing her young and death results from starvation.

Fat-Soluble Vitamine Content of Cabbage.

For these experiments cabbage as purchased on the local market was cut up fine and dried in the laboratory in an air current at room temperature. When in air-dried condition it was finally desiccated over CaCl_2 and then ground to a flour for incorporation in the ration.

The growth of rats on 5 per cent of this cabbage as the source of water-soluble vitamine shown in Chart 11 bears testimony that cabbage is not very rich in this vitamine; yet as the curves of growth are better than those where 5 per cent of alfalfa and clover (Charts 1 and 6) was the source of this vitamine, it is possible that cabbage may contain more of it. In spite of the comparatively greater initial weekly increments of growth, when failure threatened to ensue, decline in weight was prompt and decisive and moreover in two of the animals, Rats 715 and 716, observed convulsions of polyneuritis left no doubt as to the exact status

of their nutritive condition. With cabbage increased in amount to 15 per cent of the ration (Chart 12) the degree of growth and its maintenance were much improved and in three of the four animals it could be considered normal. Young were not reared.

As a source of the fat-soluble vitamine we had little success in demonstrating that cabbage is very efficient for probably a number of reasons. For one thing, cabbage when fed at high levels

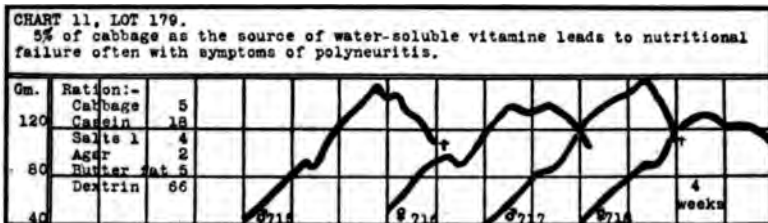


CHART 11.

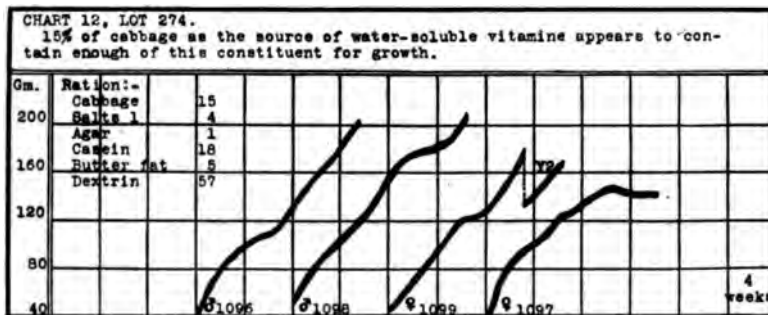


CHART 12.

is very liable to cause digestive disturbances which result in a decreased food intake with therefore a complication in the number of factors operative in the inhibition of growth. With 15 per cent of the ration constituted of cabbage, Rats 668, 669, and 670 were able to grow at a subnormal rate and maintain themselves without any of the secondary symptoms of a fat-soluble vitamine deficiency for 26 weeks which argues strongly for the presence of considerable amounts of this vitamine, yet when the amount was increased at this point by the addition of butter fat a prompt ac-

celeration in the rate of growth occurred (Chart 13). From our data we are inclined to conclude that cabbage is not a good material for the isolation of the fat-soluble vitamine, especially as with its extraction unpalatable volatile oils are removed in large amounts.

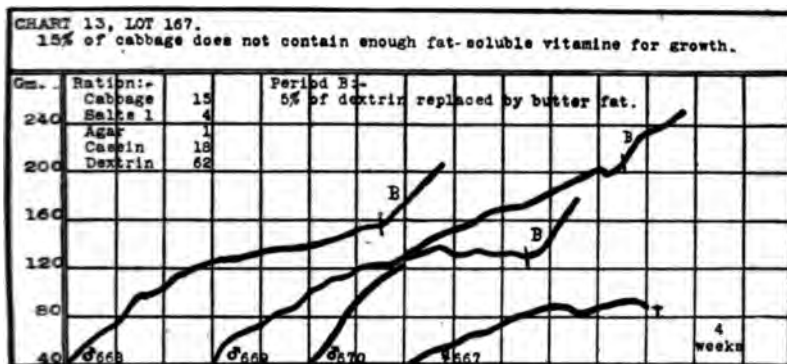


CHART 13.

Fat-Soluble Vitamine Content of Lettuce, Spinach, and Chard.

The plant materials lettuce, spinach, and chard as used in these experiments represented the stems as well as leaves of the plants as sold on the markets for household use. They were dried at room temperature in an air current and ground to a meal.

As the amounts of the above materials were limited, our observations here are not so extensive as with alfalfa and clover. Our technique was also modified in that water-soluble vitamine in all the rations was incorporated as ether-extracted wheat embryo—since from previous experience we had reason to believe that these materials were deficient in it. From the fat-soluble vitamine standpoint the use of wheat germ was entirely justifiable as with it none of this vitamine appeared to be introduced in the ration (Chart 14).

Charts 15, 16, and 17 show that 5 per cent respectively of lettuce, spinach, and chard in our basal ration furnishes enough fat-soluble vitamine for long continued though somewhat subnormal growth. Few young were produced and none was reared, but in no case

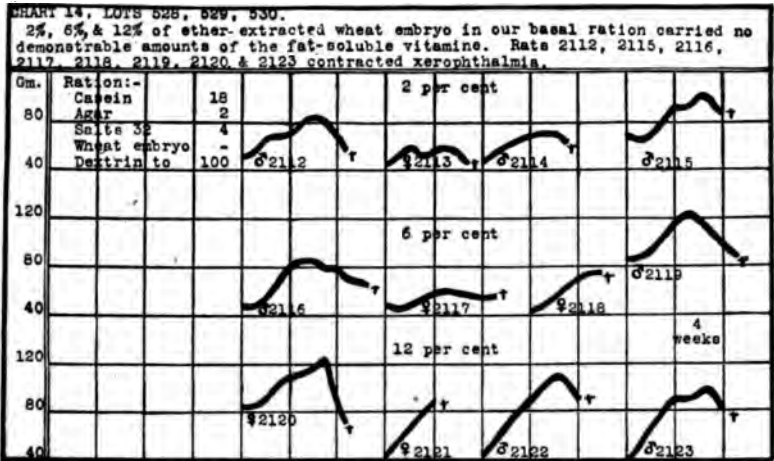


CHART 14.

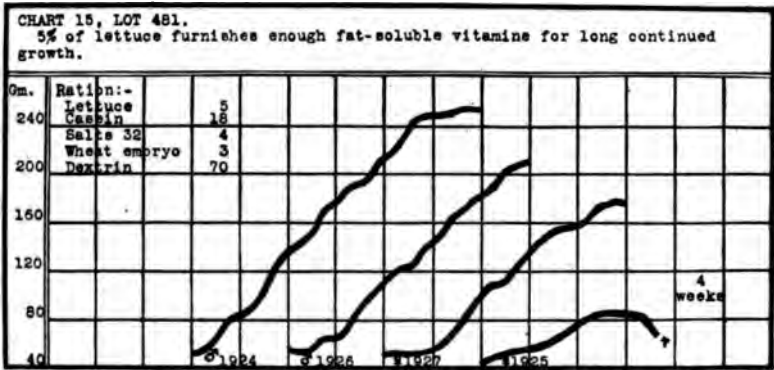


CHART 15.

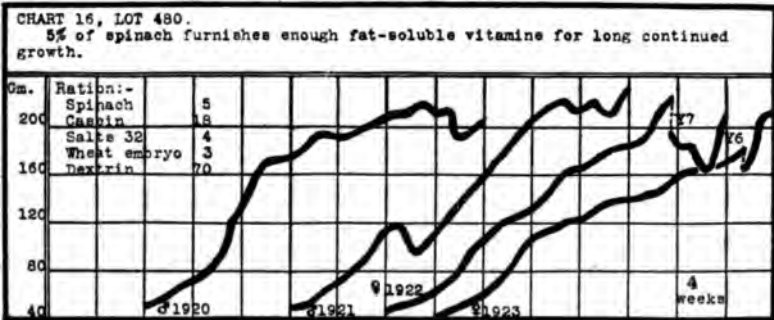


CHART 16.

was there any evidence of xerophthalmia. As judged by appearance, which is far more indicative of an animal's condition than evidence of weight relations alone, the spinach group was in the best condition and the lettuce the poorest.

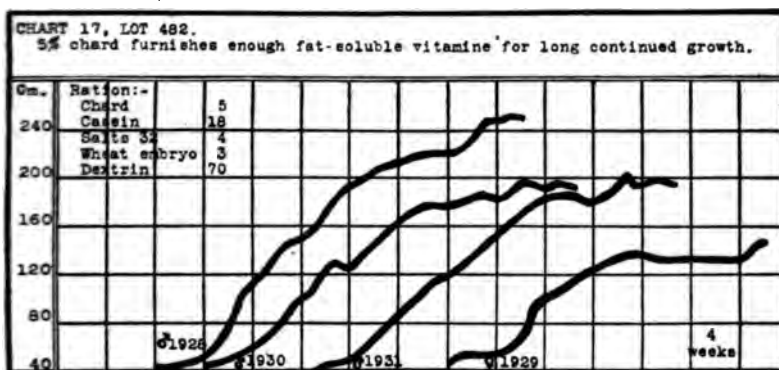


CHART 17.

DISCUSSION.

Whatever may be the reason in the physiological economy of the plant, it appears that of the plant structures the leaves are generally richest in the fat-soluble vitamine, some roots are next in order and last, at least of those investigated, are grains. In this we can see no correlation in the occurrence of the vitamine and the storage function as repeatedly enunciated by McCollum (9), but on the other hand we are assuming for our working hypothesis that where certain yellow plant pigments occur there we may look for the presence of the fat-soluble vitamine. In harmony with this, it is seen that cabbage in the head, containing little pigment, is not to be compared in physiological activity with the other leafy substances and of these latter, lettuce, also somewhat etiolated, is the poorest. Further details of this will be presented later as work now in progress is completed.

SUMMARY.

5 per cent of clover or alfalfa as the sole source of fat-soluble vitamine in a ration, when other dietary requirements are satisfied, allows normal growth and the rearing of some young.

Lettuce, spinach, and chard contain fat-soluble vitamine in amounts of similar magnitude, but of these lettuce may be the poorest.

In harmony with our theory of fat-soluble vitamine distribution and the occurrence of certain yellow plant pigments cabbage does not contain much of this vitamine as is shown by the poor results obtained even when fed at a 15 per cent level.

While 10 per cent of alfalfa and clover was inefficient, 15 per cent of either furnished enough water-soluble vitamine for normal growth; 20 per cent of clover gave still better results.

Cabbage furnished enough water-soluble vitamine when fed at a 15 per cent level.

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FAT-SOLUBLE VITAMINE.*

V. THERMOSTABILITY OF THE FAT-SOLUBLE VITAMINE IN PLANT MATERIALS.

By H. STEENBOCK AND P. W. BOUTWELL.

WITH THE COOPERATION OF MARIANA T. SELL AND E. G. GROSS.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

(Received for publication, December 23, 1919.)

In the immediately preceding paper of this series (1) we have stated the necessity of securing definite knowledge of the stability of the fat-soluble vitamine as one of the preliminaries leading to its isolation. In regard to the stability of this vitamine there appears to be no unanimity of opinion. Osborne and Mendel (2), and McCollum and Davis (3) are sponsors for the statement that it is thermostable—though the former observed one instance of its destruction by aging—while Steenbock, Boutwell, and Kent (4), and Drummond (5) have shown that it is not so stable as is generally accepted, in fact the latter was inclined to believe that he was dealing with an enzyme-like compound. While we are not inclined to subscribe to Drummond's surmisal, yet we have obtained abundant evidence that under certain conditions this vitamine is very labile, but we also have available evidence that under other conditions it may be very stable. These differences can be readily reconciled as it would be surprising indeed if variations in the stability of this substance should not occur in the very varied chemical environment in which it has been found.

The first information in this connection was presented by McCollum and Davis (3) who found extracts of egg still efficient though the yolks had previously been coagulated by heat. Osborne and Mendel (2) suspected that the impotency of commercial lard might be due to the rather drastic heat treatment to

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which the pig tissues had been subjected in the process of extraction, but this was found unwarranted as lard prepared by them in the laboratory with minimum exposure to heat was found just as inactive. Furthermore, butter fat treated with live steam for $2\frac{1}{2}$ hours was still found a source of the vitamine, though it is not stated how much was required to demonstrate that it was present in adequate amounts. Subsequently these same investigators (6) report that butter fat kept at temperatures of $8-18^{\circ}$ in the dark and also when exposed to the light did not lose its vitamine. Butter oil obtained by removal of the harder fats from butter by crystallization from warm absolute alcohol, on the other hand, gradually lost its activity even when kept at 8° and in the dark. Drummond (7) found that cod liver oil and whale oil, both of which had been obtained by steam digestion of the tissues, were still very active and therefore surmised that the vitamine was thermostable. Later, however, he substantiated the findings of Steenbock, Boutwell, and Kent (4) that its thermostability in fats such as butter is not very great. Osborne and Mendel (8) have shown that pig tissues such as liver, heart, and kidney contained the fat-soluble vitamine even though they had been dried at 90° for some hours.

In this brief résumé of the literature it is seen how little the matter of the stability of the fat-soluble vitamine has been investigated. It is believed that the data of the different investigators in this field are substantially true to fact and that the variance of opinion is due to a failure to appreciate that the reaction of destruction may be one of low velocity impeded or accelerated by secondary factors. In a ration containing twice as much vitamine as necessary for normal growth no evidence of its destruction would of course be obtained even though one-half of the amount present might have been destroyed by the treatment. In addition, there is no doubt that there obtains a great difference in the stability of the vitamine as found in different materials. This is brought out in the comparison of our previous data with those in the present paper in which are presented data on its thermostability in various plant materials.

EXPERIMENTAL.

The experimental technique used in these experiments was substantially the same as outlined in other publications of this series (1, 4, 9, 10) to which the reader is referred for details. Rats were used as the experimental animals. Shortly after weaning, they were placed on a diet which in its mixture of ingredients was known to satisfy all the dietary requirements of the animal except for the fat-soluble vitamine. The introduction of this latter component was left to that moiety of the ration which consisted of the heated plant materials. These were prepared by autoclaving them for 3 hours at approximately 15 pounds pressure—after soaking in water as they were all air-dried—and then drying them at room temperature in an air current. It was expected that by heating in the autoclave considerable amounts of the vitamine had been destroyed and with this in mind it was thought preferable to dry the materials finally at room temperature rather than in an oven. The growth curves are presented as evidence of the efficiency of the ration in furnishing the requisite amount of the fat-soluble vitamine.

In Chart 1 are presented data obtained with yellow maize, chard, and alfalfa. The maize was of the variety known as Golden Glow or Wisconsin No. 12 and was part of a lot of which the vitamine content had been determined (10). At that time reasons were given for concluding that yellow maize contains just sufficient fat-soluble vitamine to allow normal growth in some animals, but not in all. With this in mind the observed rate of growth of Rats 2503 and 2500, which is of normal proportions, has considerable significance. The somewhat subnormal rate of Rats 2501 and 2502 does not detract from this, as, by the law of averages, it is what might have been expected with animals fed on the unheated maize due to variations in the ability to grow on slightly deficient diets. It is believed safe to conclude that none of the fat-soluble vitamine of yellow maize was destroyed by autoclaving at 15 pounds pressure for 3 hours.

Chard as one of the leafy plant materials has been shown to be comparatively rich in its content of the fat-soluble vitamine (1); 5 per cent being sufficient in a low calorie ration for long continued growth. Autoclaving does not appear to result in a

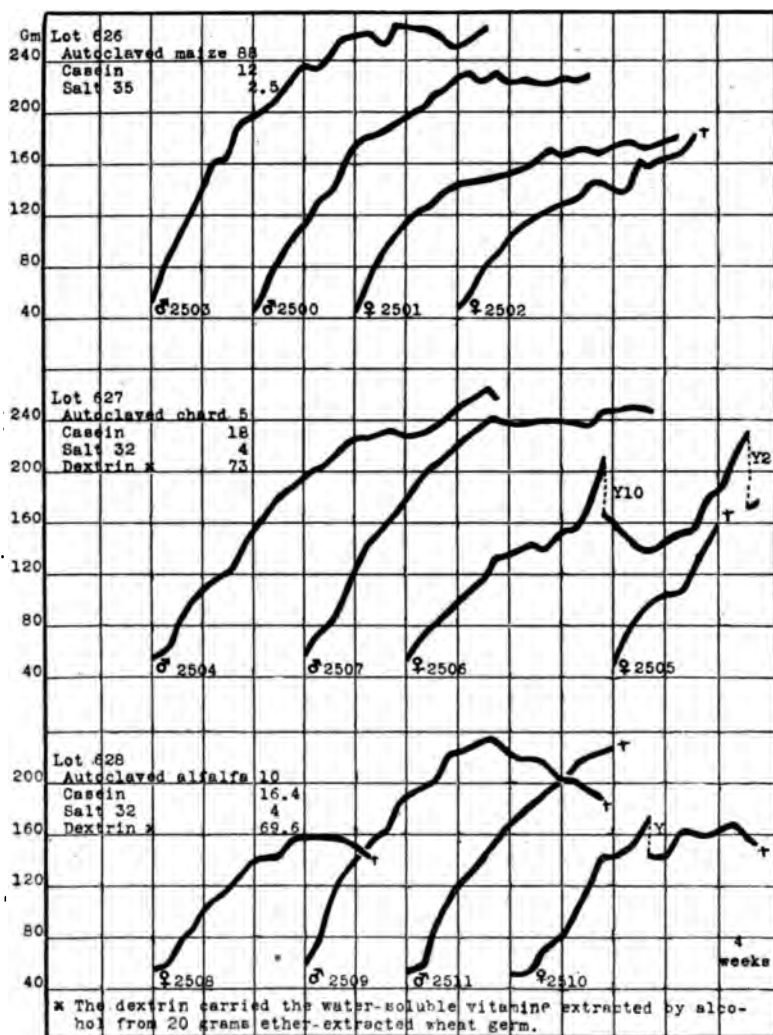


CHART 1.

diminution of its vitamine content as evident from the remarkable performance of the rats in Lot 627, Rat 2506 even rearing two out of its first litter when reduced to five young. If comparisons are made in the curves of growth of rats in this lot with those in the previous publication (1) where the unheated chard was fed, it will be noted that the latter are more nearly normal. It is believed that this discrepancy can be accounted for by the fact that the rats getting the unheated chard had water-soluble vitamine introduced in their rations as ether-extracted wheat embryo which is more depressing in its effect on growth than an alcoholic extract of the same.

In the experiment designed to bring out the effect of heat on alfalfa the autoclaved alfalfa was fed at a level at least twice as high as necessary for normal growth so that a low degree of vitamine destruction would have remained undetected. As it was, the rats grew normally on this ration for 3 to 4 weeks indicating a sufficiency of the vitamine and then declined and died. Rats 2508 and 2509, and possibly 2510, as further indications of a fat-soluble vitamine deficiency, contracted xerophthalmia before death. The condition of Rat 2511 indicated respiratory difficulty, but was not further examined. It hardly appears justifiable to conclude that a fat-soluble vitamine destruction by heat alone was responsible for this condition, especially in view of the excellent growth that had prevailed up to the time of the incidence of the disease. We reserve drawing final conclusions as either one of two possibilities appears acceptable. Either the rapid decline of the animals was occasioned by infection with an especially virulent form of the organism or organisms responsible for xerophthalmia or else while the fat-soluble vitamine was not destroyed by the heat treatment itself it was made more susceptible to destruction—possibly by liberation from combinations—by the agents operative in the aging process. What these are we do not know, but it is to be remembered that the alfalfa used in these experiments was a commercial alfalfa meal of which the history with respect to method of curing was not known. In one series of experiments carried out 2 years ago we found that the vitamine was not destroyed in the ensiling process. When green immature alfalfa was kept in a 20 gallon tub sealed air tight a considerable temperature and acidity developed in the course of 7 days. At

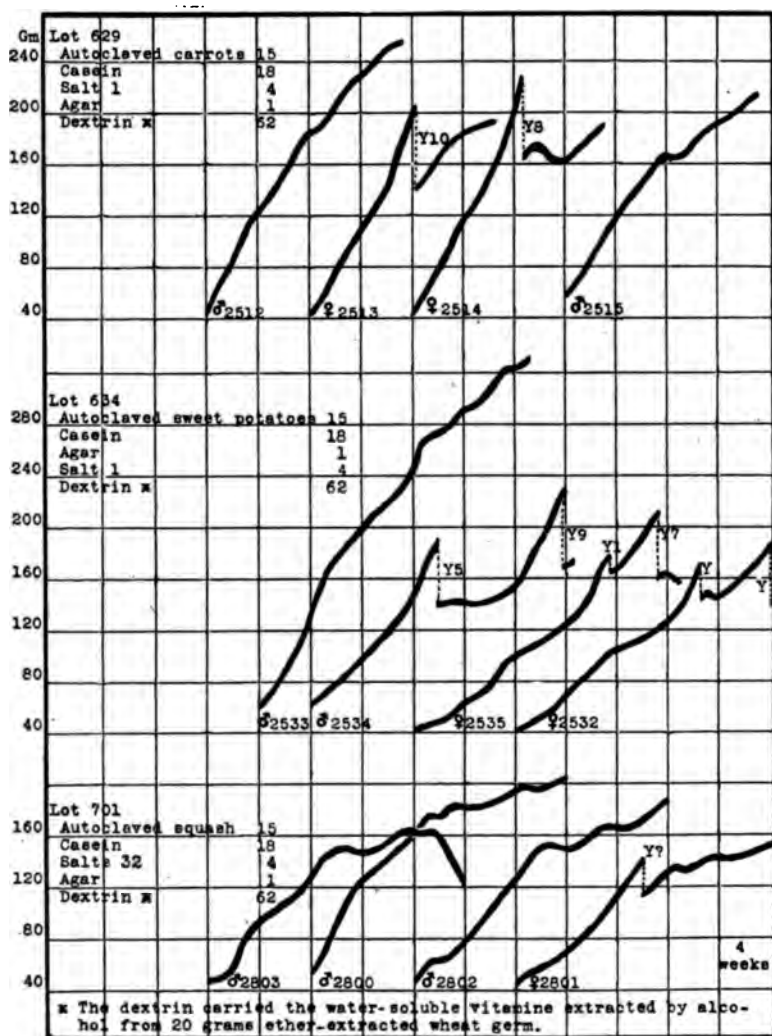


CHART 2.

the conclusion of this period the material when air-dried and fed at a 10 per cent level as the source of both the fat- and water-soluble vitamins allowed a 40 gm. male rat to attain a weight of 240 gm. in 16 weeks. Here we had a considerable acidity acting at a temperature of approximately 35° in the absence of oxygen and later—in the air drying process at room temperature—in the presence of oxygen with no complete destruction of either vitamin.

Carrots and sweet potatoes (9) both excellent sources of the fat-soluble vitamins suffer no appreciable loss of their vitamins by being autoclaved at 15 pounds pressure for 3 hours and then dried at room temperature. This is shown in Chart 2, Lots 629 and 634, where rats made very satisfactory growth when fed with

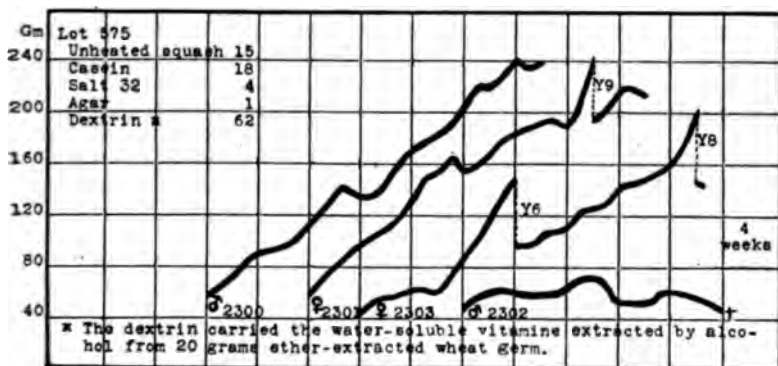


CHART 3.

these materials at 15 per cent levels. In the previous publication a fairly satisfactory degree of growth was reported with the unheated material. That the performance was here improved by the heating process is to be attributed to the reduction of the tendency to tympanites induced by the hemicelluloses in the raw materials. On both heated carrots and sweet potatoes young were successfully reared; Rat 2514, receiving carrots, raising five out of her litter of eight to an average weight of 44 gm. in 4½ weeks and Rat 2534, on sweet potatoes, raising a litter of five to an average weight of 49 gm. in 6 weeks. In view of the performance of the rats on the unheated materials we do not believe that this would have been possible if there had been any considerable destruction of the fat-soluble vitamins.

Up to the present there have not been available any data on the vitamine content of squash. If our theory of the association of certain yellow pigments and the fat-soluble vitamine (10, 11) is generally applicable in the plant kingdom it was to be surmised that the Hubbard squash which has flesh of a golden yellow would be rich in this constituent. We have not yet tested other varieties, but this particular variety was found to contain considerable amounts of the vitamine (Chart 3, Lot 575). Furthermore, like the other materials tested, its vitamine was not destroyed by autoclaving at 15 pounds pressure for 3 hours (Chart 2, Lot 701). The squash used in these experiments had been prepared, after peeling, by drying at room temperature in an air current and later over anhydrous calcium chloride. For autoclaving, it was soaked in water and after autoclaving it was again dried at room temperature. With neither the raw nor the heated squash was the growth entirely satisfactory, but that this was not due to a vitamine deficiency is attested to by the fact that in Lot 575 Rat 2303 successfully reared five young out of her litter of six to an average weight of 49 gm. in 7 weeks. Though this rate of growth was decidedly subnormal they were very active and of good appearance which would not have been the case if there had been a deficiency of the fat-soluble vitamine.

SUMMARY.

A process of heat treatment consisting of autoclaving for 3 hours at 15 pounds pressure does not destroy any of the fat-soluble vitamine as found in yellow maize. Neither does this treatment cause any noticeable destruction of the vitamine in chard, carrots, sweet potatoes, and squash as demonstrated when these materials are fed in percentages of the ration varying from 5 to 15. If some destruction occurred it was not detected, but with the amounts fed it is not believed that it could have occurred to any considerable degree, otherwise normal growth or long continued growth with reproduction would not have been possible. In the case of alfalfa the data are not decisive, but it appears that the autoclaving process in itself did not destroy the vitamine to the extent that a deficiency in the ration was thereby induced. Neither in a short time experiment was any deleterious action

of the ensiling process on the fat-soluble vitamine demonstrable. Our experiments demonstrate that the fat-soluble vitamine as found in the plant kingdom in a grain, in leaf and stem tissue, in fleshy roots, and in a cucurbitous vegetable is comparatively stable at a high temperature.

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PHOSPHORUS REQUIREMENT OF MAINTENANCE IN MAN.*

By H. C. SHERMAN.

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MATEWS, AND EMIL OSTERBERG.

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(Received for publication, December 15, 1919.)

It has for some years been inferred from the observations upon intake and output, and recently demonstrated conclusively by feeding experiments with rations otherwise adequate but deficient in one or more of the inorganic elements,¹ that considerable amounts of phosphorus are required in the normal nutrition of animals. Less emphasis than formerly is now placed upon the form of chemical combination in which the phosphorus is furnished by the food. Thus it becomes logical to discuss the amounts of phosphorus required for nutrition under different conditions without necessarily giving detailed consideration to the nature of the food intake in each case. Special interest attaches to the phosphorus requirement in man because the practise of so milling the cereal grains that the phosphorus-rich portions go to the feeding of farm animals while the parts poor in phosphorus are used for human food makes the danger of phosphorus deficiency relatively greater in human than in animal nutrition, unless it is found by adequate quantitative investigation that this tendency is fully offset by certain factors of safety of which two may be especially mentioned: (1) Growth is relatively less rapid in the child than in the young of most domesticated animals, while muscular activity at corresponding ages is probably fully as pronounced; (2) adult man may in the majority of cases be somewhat more active than the average of those adult farm animals which are

* Published as contribution No. 331 from the Department of Chemistry, Columbia University.

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131.

kept merely in a condition of maintenance or fattening as distinguished from those which serve as draft animals. For either or both of these reasons it may be that the ratio of phosphorus requirement to energy requirement is enough lower in man than in his farm animals to counterbalance the tendency to a lower percentage of phosphorus in his food, as long as his total food intake is adequate to meet his energy requirement. That grain products may constitute a smaller proportion of the entire food intake of man than of many farm animals may or may not be a factor of safety—for, while some foods such as milk and eggs are very effective supplements to the grain products in this respect (as in several others), other foods equally widely used such as butter and sugar are practically devoid of phosphorus compounds so that their inclusion in the dietary puts man at a further disadvantage in respect to his phosphorus intake.

During the past 15 years a considerable number of experiments upon the phosphorus requirement of maintenance in man have been carried out in this laboratory. The data of several of these have been included in papers previously published, sometimes with,² sometimes without,³ specific discussion of their bearing upon the present problem. The purpose of this paper is to place on record such of our experimental data as have not appeared elsewhere, and to summarize briefly the evidence of all available experiments which now seem to us to permit of direct quantitative comparison.

Experimental Data Not Recorded in Previous Papers.

Subject F, a woman of 64 kilos (studied in 1911), on a diet of bread, meat, potato, rice, prunes, butter, sugar, and tea which was estimated to furnish 0.95 gm. of phosphorus per day, showed the following average elimination in four successive periods of 3 days each which followed each other without intermission: in urine, 0.55, 0.53, 0.45, 0.50; in feces, 0.27, 0.30, 0.19, 0.23 gm.

² Sherman, H. C., Mettler, A. J., and Sinclair, J. E., *U. S. Dept. Agric., Office Exp. Stations, Bull.* 227, 1910. Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383.

³ Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 307.

respectively. It is possible that the actual intake may have been somewhat less than the analytical data indicate due to an accident which permitted partial drying of some of the foods while the samples were being taken for analysis. This, however, would in no wise affect the data of the output which we take to be an approximate indication of the phosphorus requirement of this subject (see Nos. 21 to 24 in Table I).

Subject I, a man of 61 kilos studied by A. R. Rose and the writer in 1912, took a diet consisting of bread, wheat farina, rice, egg white, milk, butter, coffee, and apple sauce, giving an intake in five successive experiments of 0.65, 0.65, 0.64, 0.56, and 0.56 gm. of phosphorus per day. These experiments followed each other without intermission and were preceded by 3 days of similar diet. The average daily output of phosphorus in each of the five experiments was as follows: in urine, 0.57, 0.60, 0.56, 0.46, 0.43; in feces, 0.26, 0.12, 0.14, 0.16, 0.09 gm. respectively. There was thus a slight minus balance at first and practical equilibrium in the latter part of the time covered by the investigation. The data of output calculated to uniform basis appear under Nos. 25 to 29 in Table I.

Subject H, a woman of 65 kilos studied in 1914, took a diet of bread, butter, wheat breakfast food, meat, potato, peanuts, and oranges with an intake of 0.61 gm. of phosphorus per day and an average daily output in each of three successive 3 day experiments as follows: in urine, 0.60, 0.68, 0.76; in feces, 0.17, 0.17, 0.19 gm. respectively (Nos. 30 to 32 in Table I).

Subject E, a man of 69 kilos studied in 1916, after 10 days on phosphorus-poor food, took during a 3 day experiment a diet of bread, butter, meat, apple, and milk furnishing 0.76 gm. of phosphorus, while the daily output for the same 3 day period was: in urine, 0.75; in feces, 0.18 gm. (No. 71 in Table I).

Subject R, a man of 80 kilos (1916), took during a period of 15 days in five experiments of 3 days each, a diet of bread, butter, and apples which furnished in the successive periods 0.56, 0.53, 0.49, 0.52, 0.57 gm. of phosphorus per day; the average daily output during each experiment was: in urine, 0.61, 0.46, 0.51, 0.43, 0.44; in feces, 0.19, 0.16, 0.19, 0.19, 0.15 gm. respectively (Nos. 72 to 76 in Table I).

TABLE I.*

Indicated Phosphorus Requirements for Maintenance per 70 Kilos of Body Weight per Day.

Experiment No.	Phosphorus.	Experiment No.	Phosphorus.	Experiment No.	Phosphorus.
	gm.		gm.		gm.
1	0.87	33†	0.84	65†	1.04
2	0.95	34†	0.85	66†	0.80
3	0.83	35†	0.71	67†	0.89
4	1.02	36†	0.74	68†	0.89
5	1.09	37†	0.74	69†	0.89
6	0.78	38†	0.69	70†	0.98
7	0.89	39†	0.76	71	0.95
8	0.68	40†	0.76	72	0.70
9	0.73	41†	0.68	73	0.54
10	1.06	42†	0.90	74	0.62
11	1.13	43†	0.89	75	0.54
12	1.12	44†	1.14	76	0.52
13	0.98	45†	1.01	77†	0.85
14	1.19	46†	1.01	78†	0.88
15	1.04	47†	0.95	79†	0.78
16	0.90	48†	1.07	80†	0.76
17	0.96	49†	1.04	81†	0.82
18	1.13	50†	1.03	82†	0.88
19	1.04	51†	0.85	83†	0.89
20	1.02	52†	0.94	84	0.77
21†	0.90	53†	0.91	85	0.79
22†	0.91	54†	0.91	86	0.69
23†	0.70	55†	0.88	87†	0.72
24†	0.80	56†	0.90	88†	0.74
25	0.96	57†	1.06	89†	0.75
26	0.83	58†	1.01	90†	0.65
27	0.81	59†	1.01	91†	0.72
28	0.72	60†	1.03	92†	0.80
29	0.60	61†	0.90	93†	0.81
30†	0.83	62†	1.08	94†	1.20
31†	0.93	63†	1.07	95†	1.17
32†	1.03	64†	0.90		
Average.....					0.88

* Experiment 1, Siven, V. O., *Skand. Arch. Physiol.*, 1901, xi, 308. Nos. 2 and 3, Gumpert, E., *Med. Klin.*, 1905, i, 1037. Nos. 4 and 5, Sherman, H. C., Mettler, A. J., and Sinclair, J. E., *U. S. Dept. Agric., Office of Exp. Stations, Bull. 227*, 1910. Nos. 6 to 9, Hämäläinen, J., and Helme, W.,

Subject Q, a man of 68 kilos (1917), took a diet of bread and milk which furnished in three successive 4 day experiments daily intakes of 0.70, 0.51, and 0.53 gm. of phosphorus respectively. The corresponding data of output were: in urine 0.58, 0.53, 0.53; in feces 0.20, 0.24, 0.12 gm. of phosphorus per day (Nos. 84 to 86 in Table I).

General Comparison of Available Data.

For convenience of comparison and discussion the data of these experiments and of all available previously published experiments which seem to be quantitatively comparable, have been calculated to the uniform basis of phosphorus output per day per 70 kilos of body weight on the same general principle as in the similar study of the protein requirement of maintenance.⁴ The data of "indicated phosphorus requirement" thus found are summarized in Table I.

It will be seen that the data of the 95 experiments range from a minimum of 0.52 to a maximum of 1.20 gm. with an average of 0.88 gm. of phosphorus per 70 kilos of body weight per day. The experiments upon men average 0.87 gm. and those upon women average 0.89 gm. per 70 kilos per day. The range of variation among the experiments is here quite similar (proportionately) to that found in a corresponding compilation of ex-

Skand. Arch. Physiol., 1907, xix, 182. Nos. 10 to 13, Berg, R., *Biochem. Z.*, 1911, xxx, 107. Nos. 14 to 20, Aron, H., and Hocson, F., *Biochem. Z.*, 1911, xxxii, 189. Nos. 21 to 24, Koch, M., and Sherman, H. C., not previously published. Nos. 25 to 29, Rose, A. R., and Sherman, H. C., not previously published. Nos. 30 to 32, Sherman, H. C., and Mathews, E. M., not previously published. Nos. 33 to 50, Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. Nos. 51 to 63, Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383. Nos. 64 to 70, Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. No. 71, Sherman, H. C., not previously published. Nos. 72 to 76, Sherman, H. C., and Osterberg, E., not previously published. Nos. 77 to 83, Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383. Nos. 84 to 86, Sherman, H. C., and Beegle, F. M., not previously published. Nos. 87 to 95, Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 307.

† Experiments upon women.

⁴ Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

periments upon the protein requirement. The same causes of variation are doubtless operative, but to different degrees in the two cases. In the study of protein requirement the earlier experiments in general tended to give high results because of failure to plan the diets properly or to continue them for a long enough time. These errors probably play less part in the phosphorus experiments here quoted since they are in the main of recent date and have been planned and carried out with these considerations in mind. As the experiments are arranged chronologically in Table I, it may be seen at a glance that there is no marked tendency to lower results among the later experiments. Probably, therefore, the question whether or not the experiment was sufficiently controlled and continued for a long enough time to test actual requirements was a less disturbing factor here than in the study of protein requirement. The function of phosphates in the maintenance of neutrality in the body may be one reason for the variations in the phosphorus output, although it has been shown⁵ that the surplus acid arising from the normal metabolism of a diet in which the acid-forming elements predominate may be eliminated in part as ammonia salt and in part as increased acidity of urine without necessarily increasing the output of phosphorus.

It is possible too that the nature of the phosphorus compounds of the intake may be of more significance than has usually been assumed during recent years. The fact that inorganic forms of phosphorus may serve to meet all the requirements of the phosphorus metabolism does not necessarily imply that all the phosphorus compounds of the intake have exactly the same quantitative efficiency in nutrition, when supplied in amounts barely adequate to meet actual needs.

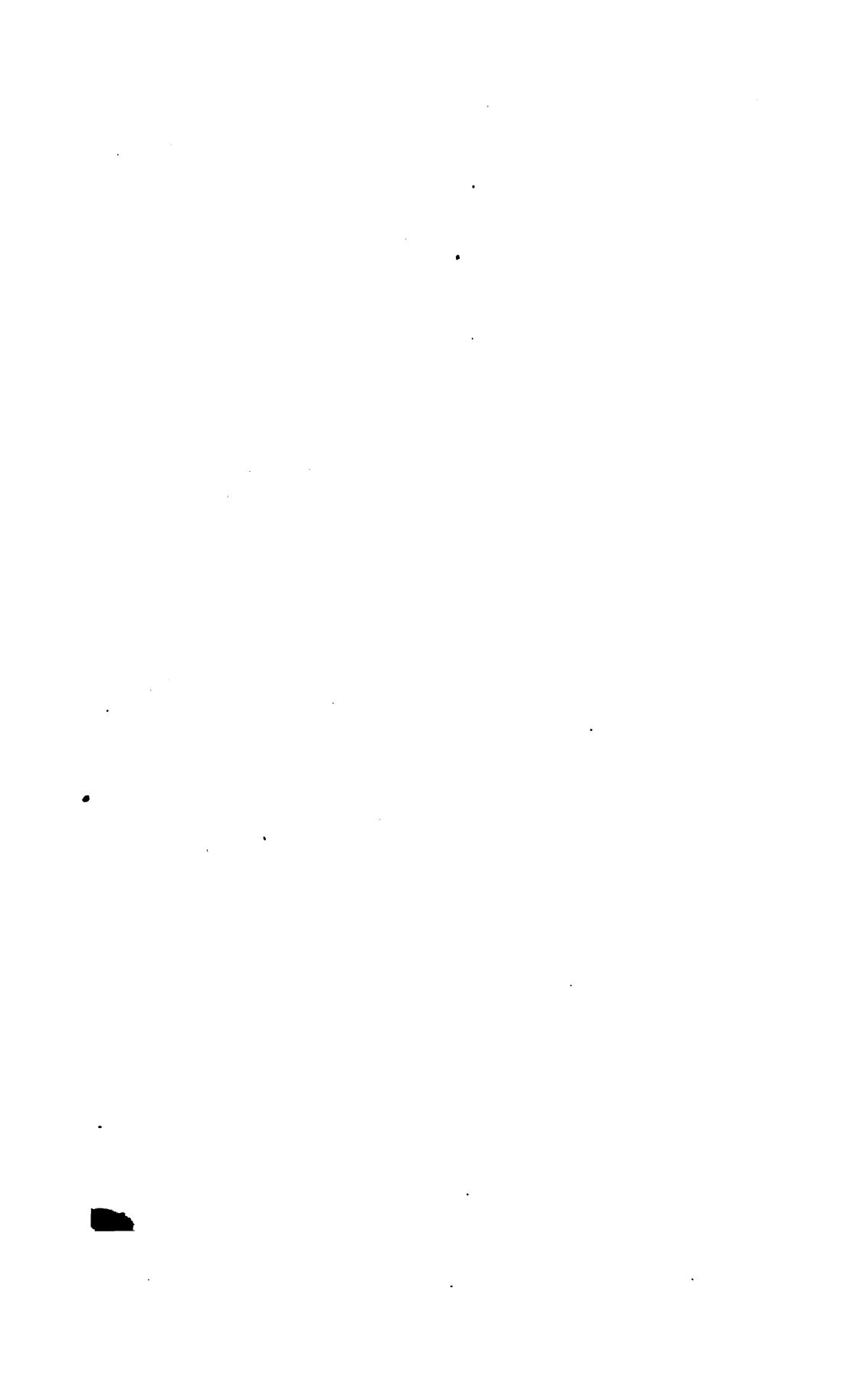
While several of the factors determining the phosphorus output remain for further quantitative investigation, we are probably justified in concluding that we now know the phosphorus requirement with about the same probable accuracy that the protein requirement is known, and that about one-fortieth to one-fiftieth as much phosphorus (reckoned as element) as of protein is required in the maintenance metabolism of man.

⁵ Sherman, H. C., and Gettler, A. O., *J. Biol. Chem.*, 1912, xi, 323.

Compared with the quantities actually required for maintenance, the average food intake of typical American households appears to provide a somewhat more liberal margin of protein than of phosphorus. Yet in a detailed study of the food supplies of 224 families or other groups of people selected as typical of the population of different parts of the United States only eight showed less than 0.88 gm. of phosphorus per man per day and in all but two of these cases the phosphorus content would have reached this figure if the food consumed (without change in its character) had been increased in amount to a level of 3,000 calories per man per day. The two cases which apparently contained less than the average actual requirement of phosphorus and would still have been thus deficient if the food had been sufficient in amount to cover the energy requirement amply were both reported from southern states. McCollum, Simmonds, and Parsons, in discussing the results of experiments upon rats with diets made up in imitation of those whose use has been described as resulting in pellagra in man, express the opinion that "it may well be that the preponderance of products of the endosperm of seeds made the phosphorus content of the diet too low."⁶ This is in addition to the inadequacy of the food as regards calcium, fat-soluble A, and certain amino-acids, which they regard as characterizing the "pellagra-producing" diets of the South. Outside of the southern regions where the food supply consists too largely of patent flour or new process (degerminated) corn-meal supplemented chiefly by sugars and fats, the danger that a freely chosen American dietary will be deficient in either protein or phosphorus does not appear serious, in the light of our present evidence, so far as the requirements of maintenance are concerned.

What quantities of phosphorus in the food will best meet the requirements of growth, pregnancy, and lactation remains to be determined.

⁶ McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 130.



THE RÔLE OF PENTOSE-FERMENTING BACTERIA IN THE PRODUCTION OF CORN SILAGE.*

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(Received for publication, December 19, 1919.)

In a previous publication¹ on the xylose-fermenting bacteria, it was noted that these organisms are easily isolated from silage, and apparently occur there in large numbers. Their optimum temperature for fermentation was shown to be about 27°C., which is approximately the average temperature found in ensiled corn. In relation to oxygen supply the pentose fermenters are most active when subjected to a low oxygen tension and will ferment xylose under anaerobic conditions. In this respect the reduced oxygen supply usually found in a silo should furnish suitable conditions for their growth.

The pentose fermenters are particularly characterized by the ease and rapidity with which they ferment pentoses, producing acetic acid and lactic acid as the chief end-products. Unpublished data on their power to ferment other sugars led to the interesting observation that from glucose they formed large quantities of alcohol, lactic acid, carbon dioxide, and small quantities of acetic acid. The fermentation of fructose by these organisms results in the formation of a characteristic product, mannitol. Dox and Plaisance² have isolated mannitol from silage in consider-

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¹ Fred, E. B., Peterson, W. H., and Davenport, A., *J. Biol. Chem.*, 1919, **xxxix**, 347.

² Dox, A. W., and Plaisance, C. P., *Iowa Agric. Exp. Station, Research Bull.* **42**, 1917.

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able quantities and its presence there is generally attributed to bacterial action.

Since xylose, or xylose-yielding substances, glucose, fructose, and other sugars, are found in abundance in green corn and since the above fermentation products are the chief chemical compounds formed in silage, a study of the relationship of these bacteria to silage production was undertaken.

For many years the relationship of microorganisms and of plant enzymes to the fermentation of silage has been studied. Some investigators have concluded that microorganisms are solely responsible for the changes in the ensilage, others that the plant enzymes are the chief causative agent. A third group of investigators has held that both factors are involved in this fermentation.³

In this article a brief report is presented of the results obtained from the inoculation of corn fodder at the time it was placed in the silo.

EXPERIMENTAL.

Numerous investigators have shown that normal silage can be produced under laboratory conditions by filling small containers with the cut corn and allowing the ensiled material to stand at a suitable temperature for a short time. The silage thus made possesses the same odor and taste, and yields on analysis the same products as silage made in the usual way. In these experiments, milk bottles closed with a one-hole rubber stopper through which passed a bent glass tube were used. The free end of the glass tube was sealed with mercury by inserting it into a test-tube containing 2 or 3 inches of mercury. Such an arrangement has been used with excellent results by Professor E. G. Hastings of the Agricultural Bacteriology Department in the experimental study of silage.

The first series was packed with rather green corn on September 5, 1919, and when filled contained 350 gm. of material in

³ For a review of the literature see Dox, A. W., and Neidig, R. E., *Iowa Agric. Exp. Station, Research Bull.* 7, 1912. Neidig, R. E., *Iowa Agric. Exp. Station, Research Bull.* 16, 1914. Sherman, J. M., *J. Bacteriol.*, 1916, i, 452. Lamb, A. R., *J. Agric. Research*, 1917, viii, 378. Hunter, O. W., *J. Agric. Research*, 1917, x, 82.

each bottle. Two bottles were used as controls and, except for the addition of 50 cc. of water, were untreated. Two others were sterilized, 50 cc. of sterilized water added, and kept as sterilized controls. Four bottles were sterilized and subsequently inoculated in duplicate with 50 cc. of a water suspension of the pentose-fermenting bacteria, Cultures 41-11 and 118-8. In order to insure growth, another set of bottles was inoculated with the same organism suspended in yeast water. The addition of this yeast water was found to be unnecessary as the bacteria grew just as well in the bottles to which no yeast water was added. The bottles were then incubated for 10 days at 27°C. During the period of incubation a strong evolution of gas was noted in all the bottles except in the sterilized uninoculated controls. In these a negative pressure was indicated by the mercury rising in the glass tube. When the bottles were opened, all except the sterilized uninoculated controls had about the same odor and taste as that of a normal silage. The contents of each bottle were put through a meat chopper to insure a uniform material, a sample was taken for the moisture determination, and as much juice expressed from the remainder as could be obtained with a strong hand press. This juice was analyzed for volatile and non-volatile acids by the usual methods; *viz.*, steam distillation for volatile acids and ether extraction of the residue from the steam distillation for non-volatile acids. The treatment of the corn fodder together with the results of the analyses is given in Table I.

An examination of the results of this table shows that in the unsterilized group there is a large production of acids and alcohol. A comparison of the products formed in the unsterilized silage with and without pentose fermenters added shows that the addition of these organisms caused a noticeable increase in volatile acid, non-volatile acid, and alcohol. These bacteria in the presence of the microorganisms commonly found in silage were able to bring about well defined differences in the chemical composition of silage.

In the sterilized group the action of these organisms is much more striking than in the raw and uninoculated group. In the absence of plant enzymes and microorganisms these bacteria brought about a decided increase in volatile and non-volatile

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acid, and also produced a small amount of alcohol. When compared with the products naturally present in the sterilized, uninoculated silage, it is evident that the pentose-fermenting bacteria are very active in fermentation of sterilized corn fodder. It is of particular interest that these bacteria in the absence of other forms are able to produce in large amounts the chief substances which are characteristic of silage. From the data, it

TABLE I.
Analysis of Silage Formed from Corn with and without Inoculation.

Treatment.	100 gm. of dry silage.			
	Moisture.	Volatile acid as acetic.	Non-volatile acid as lactic.	Alcohol as ethyl alcohol.
	per cent	gm.	gm.	gm.
Untreated corn.	80.4	0.684	6.253	2.007
Unsterilized and inoculated with Culture 41-11.	81.1	1.502	7.357	2.631
Unsterilized and inoculated with Culture 118-8.	80.4	1.480	8.389	2.552
Sterilized control.	76.2	0.240*	1.999*	
" inoculated with Culture 41-11.	78.1	1.476	4.598	0.557
" " " " 118-8.	77.2	1.207	4.387	0.311
Sterilized control plus yeast water.	76.5	0.255	1.474	
" plus yeast water inoculated with Culture 41-11.	78.1	1.370	3.454	0.543
Sterilized, plus yeast water inoculated with Culture 118-8.	78.8	1.495	4.748	0.736

* These high values are no doubt due largely to chemical changes incident to the high temperature and length of time of sterilization.

appears that the pentose fermenters either in the presence or in the absence of other microorganisms are capable of bringing about an acid fermentation of silage which is comparable with that of normal silage.

From the data for the third group, sterilized corn plus yeast water and bacteria, it will be noted that the yeast water has little if any effect on fermentation.

On September 25, another series of bottles was set up in order to study the effect of inoculation by other acid-producing bacteria

as well as the pentose fermenters. The organisms chosen were *Bacillus lactis acidi* and *Bacillus bulgaricus*. The quantity of carbon dioxide produced by the pentose fermenters was determined in this series. This was absorbed in strong alkali and the latter analyzed by means of the Van Slyke apparatus for the determination of carbon dioxide in blood and in other carbonate solutions. The results are given in Table II.

TABLE II.

Effect of Inoculation with Different Types of Acid-Producing Bacteria.

Treatment.	100 gm. of dry silage.				
	Moisture.	Volatile acid as acetic.	Non-volatile acid as lactic.	Ethyl alcohol.	Carbon dioxide.
	per cent	gm.	gm.	gm.	gm.
Untreated corn.	74.6	2.436	2.844	0.908	Not determined.
Sterilized and uninoculated.	72.9	0.352	2.002	None.	" "
Unsterilized and inoculated with <i>B. lactis acidi</i> .	67.0	1.033	2.862	0.966	" "
Sterilized and inoculated with <i>B. lactis acidi</i> .	70.8	0.339	2.305	None.	" "
Unsterilized and inoculated with <i>B. bulgaricus</i> .	70.3	1.231	3.007	"	" "
Sterilized and inoculated with <i>B. bulgaricus</i> .	68.6	0.493	Lost.	"	" "
Sterilized and inoculated with pentose fermenter No. 41-11.	71.9	1.562	3.801	0.568	0.876
Sterilized and inoculated with pentose fermenter No. 118-8.	73.0	1.335	3.475	Lost.	1.088

A study of the data of Table II clearly reveals the superiority of the pentose fermenters as compared with the other two types of bacteria in the formation of the substances usually found in silage. The latter produced little or no increase over the controls while the influence of the former is manifest in every case where they were present.

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Carbon dioxide is shown to be produced by the pentose bacteria in large quantities but no comparison with the other organisms in this respect can be made as the quantity of gas evolved from these cultures was not determined.

SUMMARY.

The results of the experiments reported in this paper indicate that the pentose-fermenting bacteria are capable of bringing about decided changes in raw or in sterilized corn tissue. When added to raw corn fodder, these organisms are able to compete with the fermentation processes which normally occur. In sterilized silage the pentose fermenters develop rapidly and produce the substances commonly found in good silage; *viz.*, acetic acid, lactic acid, ethyl alcohol, and carbon dioxide.

From the standpoint of temperature, oxygen supply, and fermentable compounds, silage offers a suitable medium for the growth of the pentose fermenters. The authors feel that the results indicate that these bacteria play an important part in the formation of corn silage.

NOTE ON THE HYDROGEN ION CONCENTRATION OF THE HUMAN DUODENUM.

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(Received for publication, December 29, 1919.)

It has long been known that the stomach is acid in comparison to the ileum, and it follows from this that the reaction of the duodenum must be influenced by the opening of the pylorus. According to Cannon, the opening of the pylorus is controlled by the acidity of this region, but it has been shown by a number of workers, most recently by Luckhardt, Phillips, and Carlson, that the motor phenomena of the stomach have a great influence on the passage of its contents through the pylorus. Perhaps the following description is nearly correct: Acid influences the tone of the pylorus but whenever this is less than the tone of the stomach or duodenum the passage of fluid may occur. The passage must necessarily be toward the region of lesser tone and is not always in the same direction, as shown by the fact, known for a long time, that the stomach contents may occasionally be bile-stained. Since the chyme is acid and the pancreatic juice alkaline, the relative rate of the flow of these two into the duodenum must influence its reaction.

In 1915 Dr. John P. Schneider gave one of us two samples of human duodenal contents which were found to be of pH 1.5 and 7.61. He had been removing the duodenal contents from many of his patients with an Einhorn duodenal tube in order to estimate the bile pigments. He allowed the duodenal contents to syphon out of the tube and found that the flow was intermittent. Sometimes the fluid spurted out and it was then acid to litmus whereas otherwise it was not acid to litmus (paper). It was shown by McClendon that the duodenum of the infant, although always acid, was very variable, the reaction sometimes approach-

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ing neutrality (pH = 6.3). Long and Fenger, using the duodenal tube, observed great variation in the adult duodenum (pH = 3.80 to 7.81). The technique used in the present paper differs from theirs only in the use of the hydrogen electrode described by McClendon and Magoon.

The Einhorn duodenal tube was swallowed by one of us (F.J.M.) and allowed to descend to the first mark, then carefully lowered to the second mark. The subject was then placed on his right side on a couch, with his hips elevated. An average of about 3.5 hours was required before the end of the tube with the lead

TABLE I.

Meal.	Food taken.	pH
Breakfast.	Hot cakes, toast, coffee.	3.80
"	Bacon, rice, coffee.	3.20
"	Corn flakes and cream, eggs, toast.	6.98
Luncheon.	Beef, potatoes, tomatoes, pie.	4.60
"	Pork, " eggplant, cake.	5.00
Breakfast.	Hot cakes, pineapple sauce, coffee.	7.21
"	Toast, raspberry sauce.	7.40
"	Ham, eggs, toast, coffee.	7.54
Luncheon.	Beef, potatoes, corn, ice-cream.	7.00
"	" " bread, melon, iced tea.	7.82
"	Potatoes, carrots, pie, cake, ice-cream, milk.	7.60
" *	Beef, onions, potatoes, pie, cake followed by bismuth.	7.54

* The fluoroscope was used in conjunction with bismuth and the duodenal tube after the digestion of the food was well under way.

weight seemed to be in the duodenum. The degree of traction on the tube was at first used as an index of its passage of the pylorus, but since some of the samples were acid we began to doubt that the pylorus had been passed, and confirmed this by use of the fluoroscope, for which our thanks are due the University Hospital.

In Table I the determinations that were acid and those that were alkaline are separated for comparison. We have no doubts that the acid samples came from the duodenum, since all samples were taken about 3 or 4 hours after the last meal and it has been shown by McClendon that the pH of the stomach at this time was between 1 and 2.5 in all normal individuals examined, whereas

the acid samples reported in Table I have a pH between 3.2 and 6.98.

After the position of the lead weight on the end of the duodenal tube in the duodenum was shown by means of the fluoroscope, it

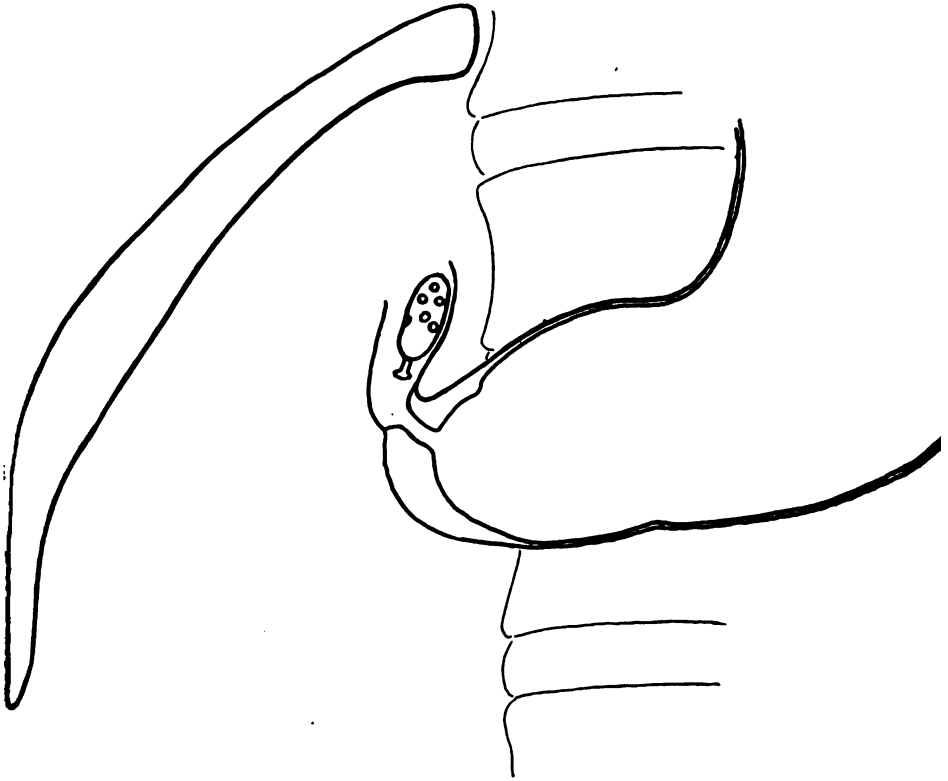


FIG. 1.

was drawn down toward the pylorus by traction on the tube and a photograph taken. A tracing from the x-ray plate is shown in Fig. 1. The dim edges of shadows in the plate were sharpened by free-hand drawing in making the tracing.

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CONCLUSIONS.

The reaction of the duodenum between 3 and 4 hours after meals was usually found to fluctuate around the neutral point, but the extreme range on the acid side was greater than on the alkaline side, possibly due to the spurting of gastric contents into the duodenum.

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STUDIES OF ACIDOSIS.

XV. CARBON DIOXIDE CONTENT AND CAPACITY IN ARTERIAL AND VENOUS BLOOD PLASMA.

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(Received for publication, December 29, 1919.)

According to the facts at our disposal, the bicarbonate content of the arterial blood plasma appears to be the blood figure most accurately indicating the alkaline reserve of the body fluids as a whole (Van Slyke and Cullen, 1917; Palmer and Van Slyke, 1917). In man it has heretofore been necessary to depend upon bicarbonate determinations in the venous blood as the closest practicable approximation to the arterial bicarbonate. In practice this has been estimated by determining the carbon dioxide capacity; that is, the CO_2 content of the plasma after saturation with air containing CO_2 at approximately the tension of normal alveolar air (Van Slyke and Cullen, 1917). The reliability of such determinations for the diagnosis of acidosis in metabolic diseases may be considered as demonstrated by the tests to which the method has been put in various laboratories, but it nevertheless remains desirable to compare the results thus obtained on the venous plasma with the actual arterial bicarbonate. The utilization of a technique for arterial punctures (Stadie, 1919) has rendered it possible to make this comparison in a series of patients, and the results are presented in this paper. Altogether thirty individuals were studied, most of whom had bronchopneumonia or lobar pneumonia of varying degrees of severity. A few normal individuals are included.

Methods.

The arterial blood was obtained as previously outlined (Stadie, 1919). The venous blood was taken without stasis, and, as a rule, 1 to 3 minutes after the arterial; both arterial and venous bloods were collected out of contact with air under albolene.

The CO₂ content and capacity were determined by the methods of Van Slyke (1917) and Van Slyke and Cullen (1917) respectively. The blood, collected under albolene to prevent loss or gain of CO₂, was centrifuged, and 1 cc. samples of the plasma were withdrawn and discharged into the cup of the Van Slyke apparatus under a little ammonia to prevent escape of CO₂. After thus determining the CO₂ content, the remaining plasma was saturated with air containing approximately 5.5 volumes per cent of CO₂, and the CO₂ capacity then determined.

In calculating CO₂ *capacity* (by Table I, Van Slyke and Cullen, 1917) the CO₂ physically dissolved (H₂CO₃) is subtracted, so that the results represent only CO₂ bound as bicarbonate.

In calculating the CO₂ content, however (by Table I, Van Slyke, 1917), no subtraction for physically dissolved CO₂ is made, and the data represent total CO₂ from NaHCO₃ and H₂CO₃ together. The free CO₂ in normal arterial plasma is about 3 volumes per cent; that is, a CO₂ content of 65 volumes per cent represents approximately 62 volumes per cent of bicarbonate CO₂ and 3 volumes per cent of free carbonic acid CO₂.

The arterial oxygen unsaturation, or the percentage of hemoglobin in the arterial blood not combined with oxygen, was calculated as described by Lundsgaard (1918) using Van Slyke's method (1918) for the oxygen determinations. The oxygen content of the arterial blood was determined, and then a portion was thoroughly aerated, and the oxygen capacity determined.

$$\text{Per cent oxygen unsaturation} = \frac{\text{O}_2 \text{ capacity} - \text{O}_2 \text{ content}}{\text{O}_2 \text{ capacity}} \times 100.$$

In normal individuals at rest the arterial unsaturation averages about 5 per cent, 95 per cent of the hemoglobin in the arterial blood being saturated with oxygen. Figures for the unsaturation higher than 8 per cent indicate incomplete oxygenation of the arterial blood.

The results are given in Table I. In a few cases the arterial CO₂ content equals or slightly exceeds the venous. This may have been due to the short differences in time between the drawing of arterial and venous bloods, or to a summation of experimental errors in the two determinations. The latter factor cannot be excluded because in many cases the amount of material did not permit analyses in duplicate. It is believed,

TABLE I.

Case No.	Arterial plasma.		Venous plasma.		A	B	C	Arterial oxygen unsaturation.
	CO ₂ content.	CO ₂ capacity.	CO ₂ content.	CO ₂ capacity.	Arterial content	Arterial content	Arterial content	
	Arterial capacity	Venous capacity	Arterial content	Venous content	Arterial content	Venous content	Arterial content	
	vol. per cent	vol. per cent	vol. per cent	vol. per cent				per cent
3		60.3		60.3				11.9
	65.7	67.2	66.6	70.0	0.978	0.939	0.968	9.0
	69.9		74.6	75.8		0.922	0.937	6.0
	70.8	72.9	71.1	72.9	0.971	0.971	0.996	0
6	65.2		71.8	69.2		0.942	0.942	
8			67.3	73.7				8.1
9	50.3	56.8	50.8	54.9	0.886	0.916	0.990	68.2
10	69.0	73.0	68.4	71.6	0.945	0.964	1.009	4.6
11	57.3	62.4	59.2	64.9	0.918	0.883	0.968	10.1
	62.6	67.5	66.0	68.9	0.928	0.909	0.948	8.9
12	59.5	66.5	63.8	69.4	0.895	0.857	0.933	6.3
	56.2	61.7	60.1	64.6	0.911	0.870	0.935	13.7
13	63.5	66.2	62.5	66.2	0.959	0.959	1.016	2.8
	61.9	62.1	64.7	65.0	0.997	0.952	0.957	7.5
14	63.2	70.0			0.903			20.7
15	55.5	62.3	60.9	66.3	0.891	0.837	0.911	7.8
	63.1	65.3	67.4	69.1	0.966	0.913	0.936	7.3
16	55.8	59.4	59.7	66.7	0.939	0.837	0.935	14.1
17	59.6	62.2	60.5	62.2	0.958	0.958	0.965	16.3
	69.1	71.6	77.6	78.1	0.965	0.885	0.891	11.5
18	56.0	63.3	57.8	63.3	0.885	0.885	0.969	16.6
	61.4	64.0	59.0	64.0	0.959	0.960	1.041	16.5
			63.1	70.8				38.2
19	63.9	68.4	65.8	68.4	0.934	0.934	0.971	7.9
	57.1	76.5	61.8	76.0	0.746	0.751	0.924	0
20	55.7	62.0		61.1	0.898	0.912		13.4
22	63.3	70.8	71.1	75.5	0.894	0.838	0.891	19.5
	46.8	51.9	51.5	50.0	0.902	0.936	0.909	25.1
23	66.8	62.1	69.7	69.6	1.076	0.960	0.959	15.1
			67.2	78.1				8.9
24	64.5	66.2	71.2	68.1	0.974	0.947	0.906	2.4
25			54.9	50.9				44.1
26	63.7		71.9				0.886	5.2
30	50.2	52.6	57.3	65.8	0.954	0.763	0.876	24.9
33	55.5	60.0	58.4	57.2	0.925	0.970	0.950	23.4
34	58.8	61.4			0.958			14.9
	53.3	57.2	53.3	59.2	0.932	0.900	1.000	33.5
	56.7	58.8			0.964			25.9
	56.1	57.8			0.971			10.7
38	56.2		60.6	63.2		0.889	0.927	8.4
39	57.4	61.0	59.3	66.8	0.941	0.859	0.968	14.5
40	59.8	59.5			1.005			
41	55.7	61.0			0.913			
42	50.8	51.4			0.988			
Average.....					0.938	0.904	0.950	

however, that the number of determinations is sufficient to rule out the influence of such factors on the results viewed as a whole. The latter seem to justify the following conclusions.

CONCLUSIONS.

1. The carbon dioxide capacity (NaHCO_3) of the venous blood plasma in man determined by the technique of Van Slyke and Cullen (1917) parallels the arterial plasma carbon dioxide content ($\text{NaHCO}_3 + \text{H}_2\text{CO}_3$), which it exceeds on the average by about one-tenth. As the arterial CO_2 is 95 per cent due to bicarbonate, the above results mean that the venous plasma carbon dioxide capacity parallels the arterial plasma bicarbonate, averaging about 115 per cent as great (Column B of Table I).

2. The carbon dioxide *content* of plasma from venous blood drawn without stasis parallels the arterial slightly more closely than does the venous carbon dioxide capacity and averages 105 per cent of the arterial content (Column C).

3. Consequently, for estimating the alkaline reserve in man, the venous plasma may be used for CO_2 determination directly, without resaturation with carbon dioxide, if the blood is centrifuged and the plasma brought to analysis without opportunity for escape of CO_2 .

4. Even when pulmonary conditions in pneumonia become so unfavorable for gas exchange that the arterial blood is incompletely oxygenated (high oxygen unsaturation), the arterial and venous carbon dioxide values are not increased above the usual normal levels. This might be expected from the fact shown by Krogh and Krogh (1910) that the lungs maintain approximate equality of carbon dioxide tension between arterial blood and alveolar air much more readily than they maintain equality of oxygen tension.

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EFFECT OF CALCIUM ON THE COMPOSITION OF THE EGGS AND CARCASS OF LAYING HENS.

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(Received for publication, December 4, 1919.)

In connection with certain studies concerning the growth of the White Leghorn hen, an experiment was planned to determine the effect produced by grit, oyster shell, and limestone on the composition of the eggs laid when fed in connection with an ordinary ration used for laying hens; and also to determine what would be the ultimate effect on the body of the hens of the continued laying of eggs in the absence of calcium other than that contained in the food. In other words, it was proposed to determine the composition of eggs of hens whose supply of calcium was limited to that contained in a dry mash and mixed grains (having a low calcium content) as compared with the composition of the eggs from hens receiving all the calcium they might desire from oyster shell and ground limestone. It also seemed important to determine to what degree the continued laying of eggs, on a limited intake of calcium, would lower the calcium content of the carcass of the hen before she should stop laying. The corresponding distribution of magnesium and phosphorus was also to be determined.

With these ends in view, forty pure bred, White Leghorn pullets were selected from the same incubator hatching and divided into four lots of ten each, having approximately the same development and vigor. These were placed in four separate hen houses which were identical in every way, and during the entire experiment the pullets were not allowed access to the ground, thus eliminating any possible chance of their obtaining inorganic material from undesired sources. These lots were designated Nos. 1, 2, 3, and 4 and all received the same ration; namely, a dry mash composed of 6 parts corn-meal, 3 parts bran, 3 parts middlings,

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5 parts meat meal, and 5 parts charcoal; and a grain mixture of 16 parts wheat, 16 parts cracked corn, and 8 parts oats. These foods were supplemented in Pen 2 with grit, in No. 3 with grit and oyster shell, and in No. 4 with grit and limestone; whereas Pen 1 received no additional mineral matter. The percentages of calcium, magnesium, and phosphorus in the foods and the supplemental mineral material are given in Table I, calculated as the oxides.

At the beginning of the experiment, December 1, 1918, separate analyses were made of the shell and of the contents of an average egg. Also a representative pullet was killed and the head, skin, feathers, feet, and intestines with the contents of the gizzard were discarded, to eliminate any extraneous matter. The two large upper bones (femur and tibia) of both legs were dissected

TABLE I.
Analysis of the Materials Fed.

Material.	Crude ash.	Calcium oxide (CaO).	Magnesium oxide (MgO).	Phosphorus pentoxide (P ₂ O ₅).
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Grain mixture.....	2.37	0.0013	0.0006	0.0078
Mash "	7.75	0.0192	0.0024	0.0182
Grit.....		1.00	0.82	0.09
Oyster shell.....		51.85	0.37	0.12
Limestone.....		47.95	1.00	0.18

out and, after being freed from adhering material, were analyzed separately from the remaining part of the carcass. During the progress of the experiment, when marked visible changes appeared in the hens, an egg was obtained on the same day from each of the four lots and, after being carefully cleaned with distilled water, the shells and their contents were separately analyzed for calcium, magnesium, and phosphorus. Also, whenever a hen broke down, she was killed and analyzed as stated above.

As the experiment advanced it was noticed that the general condition of Lots 1 and 2 was not so good as that of Lots 3 and 4 which received the calcium supplement. It will be seen in Table II that deaths occurred in each lot except Lot 3; they occurred from various causes which are not of such a character as would result directly from lack of proper nourishment. In Lot 1 there

was no instance of a complete breakdown similar to those that occurred in Lot 2 on March 9th and May 20th. It seems most probable that this breaking down in Lot 2 was due to individual weakness rather than to the grit supplementing their ration, which was the only point in which the ration differed from that of Lot 1. The average number of eggs laid per hen, per month, was approximately the same in Lots 1 and 2, while in Lot 4 the average was 13.5 per cent greater than that of Lot 3, which may or may not be attributed to individual variation.

In all lots, March and April seem to be the periods of greatest average egg production and while this production was less in all

TABLE II.
Mortality and Egg Record.

Lot No.	December.		January.		February.		March.		April.		May.			Total eggs laid in 6 months.	Average No. of eggs per hen.			
	No. of hens.	Total eggs per hen.	No. of hens.	Total eggs per hen.	No. of hens.	Total eggs per hen.	No. of hens.	Total eggs per hen.	No. of hens.	Total eggs per hen.	No. of hens.	Total eggs per hen.						
1	10	0.0	10	16.1	10	54.5	9*	80	8.9	9	120	13.3	9	21	2.3	291	31.5	
2	10	20.2	9†	27.3	8†	51.6	7†	71	10.1	7	41	5.8	7	37	5.3	229	30.6	
3	10	0.0	10	20.2	10	86.8	6	10	17.1	10	122	12.2	10	89	8.9	488	48.8	
4	10	30.3	10	43.4	9*	73.8	1	9	143	15.9	9	137	15.2	8*	102	12.8	491	56.4

* Cause of death unknown.

† Death caused by chicken pox.

‡ This hen was killed after breaking down and was analyzed; see Table V.

lots during the month of May than it was in March or April, yet it can plainly be seen that the lack of mineral matter increased the difference.

As will be seen in Tables III and IV, which give the analyses of the egg shells and their contents, an egg was analyzed December 1, which marked the starting of the experiment, and on February 12, or approximately 10 weeks later, an egg was analyzed from each lot, since it was noticed at this time that the general appearance of Lots 1 and 2 did not equal that of Lots 3 and 4. This difference became more exaggerated as time passed until, on March 8th, a hen in Lot 2 broke down. She was unsteady and remained in a squatting position, unless disturbed. On the day

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following, the hen could not stand and, since her appetite was practically gone, she was killed and analyzed as described elsewhere. The results of these analyses are shown in Table V. At this point an egg was obtained from each lot and analyzed.

TABLE III.
Analyses of Shells of Eggs from All Lots.

Date..	Lot No.	Total shell.	Total ash.		Calcium, as CaO, in ash.		Magnesium, as MgO, in ash.		Phosphorus, as P ₂ O ₅ , in ash.	
		gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent
Dec. 1, 1918	Normal.	4.702	2.753	58.55	2.720	98.10	0.019	0.71	0.019	0.70
Feb. 12, 1919	1	5.129	2.930	57.13	2.850	97.25	0.018	0.62	0.020	0.69
	2	4.660	2.586	55.47	2.525	97.65	0.016	0.62	0.013	0.51
	3	5.742	3.083	53.69	3.030	97.30	0.023	0.77	0.020	0.65
	4	5.853	3.191	54.53	3.129	98.05	0.019	0.61	0.023	0.74
Mar: 8, "	1	4.685	2.541	54.25	2.475	97.40	0.018	0.74	0.015	0.59
	2	3.910	2.119	54.20	2.093	98.80	0.012	0.61	0.011	0.54
	3	5.725	3.172	55.41	3.123	98.45	0.019	0.63	0.012	0.40
	4	4.563	2.522	55.28	2.482	98.40	0.013	0.54	0.018	0.73
" 22, "	1	3.608	1.934	53.61	1.895	98.00	0.013	0.72	0.012	0.67
	2	3.775	2.035	53.92	1.986	97.95	0.017	0.87	0.011	0.57
	3	5.879	3.222	54.82	3.164	98.20	Lost.	Lost.	0.013	0.41
	4	5.903	3.189	54.03	3.119	97.80	0.024	0.76	0.022	0.70
May 20, "	1	*								
	2	3.050	1.604	52.61	1.577	98.30	0.013	0.81	0.009	0.62
	3	4.664	2.497	56.12	2.447	98.00	0.022	0.89	0.015	0.60
	4	5.269	2.956	56.11	2.885	97.60	0.027	0.93	0.028	0.97
June 1, "	1	2.949	1.564	53.03	1.524	97.50	0.013	0.89	0.009	0.60
	2	2.797	1.542	55.14	1.500	97.20	0.015	1.03	0.013	0.86
	3	3.233	1.954	60.46	1.918	98.15	0.015	0.80	0.017	0.90
	4	4.320	2.519	58.32	2.471	98.10	0.022	0.90	0.016	0.65

* The shell of the egg obtained from Lot 1 on May 20 was so thin that it broke in handling.

On March 22, May 20, and June 1 an egg was obtained from each lot and analyzed and, since, on June 1, the hens in Lots 1 and 2 were in a state bordering on a general breakdown and had practically ceased laying eggs, the experiment was brought to a

close and an average hen from each lot was killed and analyzed. In Table VI will be found the analyses of the carcasses and leg bones of hens from each of the four lots, made 6 months after the start of the experiment.

TABLE IV.
Analyses of the Contents of Eggs from All Lots.

Date.	Lot No.	Total con- tents.	Total ash.		Calcium, as CaO, in ash.		Magnesium, as MgO, in ash.		Phosphorus, as P_2O_5 , in ash.	
		gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent
Dec. 1, 1918	Normal.	43.216	0.410	0.95	0.033	8.12	0.006	1.46	0.164	40.05
Feb. 12, 1919	1	44.095	0.423	0.96	0.034	8.05	0.005	1.38	0.157	37.20
	2	41.711	0.354	0.85	0.030	8.31	0.005	1.58	0.155	44.00
	3	47.022	0.456	0.97	0.038	8.50	0.007	1.57	0.181	39.77
	4	39.612	0.380	0.96	0.032	8.55	0.005	1.33	0.161	42.40
Mar. 8, "	1	49.702	0.412	0.83	0.030	7.44	0.006	1.45	0.164	39.71
	2	52.110	0.437	0.84	0.035	8.04	0.004	1.78	0.144	33.00
	3	44.201	0.419	0.95	0.033	7.93	0.004	1.13	0.184	44.04
	4	42.846	0.372	0.87	0.028	7.51	0.005	1.34	0.164	44.11
" 22, "	1	37.800	0.378	1.00	0.030	7.98	0.004	1.12	0.141	37.51
	2	44.315	0.398	0.90	0.025	6.45	0.003	0.81	0.148	37.31
	3	52.080	0.479	0.92	0.039	8.34	0.005	1.22	0.194	40.50
	4	52.413	0.487	0.93	0.038	8.05	0.004	0.87	Lost.	Lost.
May 20, "	1	*								
	2	39.788	0.338	0.85	0.023	6.86	0.005	1.52	0.125	37.20
	3	45.042	0.427	0.95	0.035	8.32	0.005	1.37	0.206	48.20
	4	44.215	0.415	0.97	0.034	8.31	0.004	1.12	0.206	49.60
June 1, "	1	37.161	0.308	0.83	0.020	6.50	0.003	1.14	0.121	39.50
	2	35.083	0.287	0.82	0.021	8.10	0.003	1.06	0.113	46.00
	3	37.521	0.352	0.94	0.037	10.80	0.004	1.40	0.162	46.10
	4	51.019	0.474	0.93	0.033	7.19	0.005	1.09	0.233	49.20

* The shell of the egg obtained from Lot 1 on May 20th was so thin that it broke in handling.

In analyzing the separate portions of the ash of the materials under consideration, the phosphorus was determined by the method of the Association of Official Agricultural Chemists,¹

¹ Wiley, H. W., Official and provisional methods of analysis, U. S. Dept. Agric., Bureau of Chemistry, Bull. 107, revised, 1912.

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TABLE V.
Analyses of Normal Hen and a Hen That Had Broken Down in Lot 2.

Date.	Lot No.	Part analyzed.	Total weight of part analyzed.		Total ash.		Calcium, as CaO, in ash.		Magnesium, as MgO, in ash.		Phosphorus, as P ₂ O ₅ , in ash.	
			gm.	per cent.	gm.	per cent.	gm.	per cent.	gm.	per cent.	gm.	per cent.
Dec. 1, 1918	Normal.	Carcass. 4 leg bones.	727.00	2.79	20.305	2.79	7.563	37.25	0.213	1.05	7.750	38.17
			24.34	29.70	7.235	29.70	3.766	52.05	0.048	0.67	2.841	39.27
Total.....			751.34		27.540		11.329		0.261		10.591	
Mar. 9, 1919	Broken down in Lot 2.	Carcass. 4 leg bones.	513.50	2.91	14.934	2.91	5.354	35.85	0.158	1.06	6.318	42.31
			19.05	19.86	3.783	19.86	1.920	50.75	0.024	0.64	1.494	39.50
Total.....			532.55		18.717		7.274		0.182		7.812	

TABLE VI.
Analyses of Carcasses and Leg Bones of Hens from All Lots, at the End of the Experiment, June 1, 1919.

Date.	Lot No.	Part analysed.	Total weight of part analysed.		Total ash.		Calcium, as CaO, in ash.		Magnesium, as MgO, in ash.		Phosphorus, as P ₂ O ₅ , in ash.	
			gm.	per cent.	gm.	per cent.	gm.	per cent.	gm.	per cent.	gm.	per cent.
June 1, 1919	1	Carcass.	629.00	3.69	23.269	3.69	7.213	31.00	0.235	1.01	7.574	32.55
		4 leg bones.	32.00	26.83	8.586	26.83	4.602	53.60	0.059	0.69	3.484	40.58
	Total.....		661.00		31.855		11.815		0.294		11.058	
	2	Carcass.	552.00	2.76	15.274	2.76	4.643	30.4	0.139	0.91	4.803	31.45
		4 leg bones.	24.90	18.10	4.509	18.10	2.371	52.6	0.025	0.57	1.739	38.58
	Total.....		576.90		19.783		7.014		0.164		6.543	
	3	Carcass.	836.00	3.23	27.020	3.23	10.470	38.75	0.375	1.38	10.851	40.16
		4 leg bones.	33.00	34.36	11.338	34.36	6.240	55.04	0.329	2.91	4.382	38.66
	Total.....		869.00		38.358		16.710		0.704		15.233	
	4	Carcass.	731.00	3.61	26.400	3.61	9.570	36.25	0.398	1.51	9.240	35.00
		4 leg bones.	32.00	25.02	8.005	25.02	4.419	55.20	0.249	3.11	3.216	40.18
	Total.....		763.00		34.405		13.989		0.647		12.456	

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while calcium and magnesium were determined according to the method of McCrudden.²

From Tables III and IV it will be seen that there is little variation in the percentages of ash, calcium, magnesium, and phosphorus in the egg shells and the contents of the eggs in all four lots during the 6 months over which the experiment lasted. However, there was a decided tendency in Lots 1 and 2 for the total weights of the shell to become smaller as the experiment advanced; in other words, the shell became progressively thinner. On May 20 the shell of the egg obtained from Lot 1 was so thin that it broke in handling and was lost. In the case of Lots 1 and 2, on June 1, the eggs were handled in cotton-padded boxes, to prevent breaking. In no case was a shell-less egg obtained from these four lots.

In Table V we see that the total weight of the parts of the hen which broke down on March 9 was 218.79 gm. less than the normal hen that was over 3 months younger. One of the most interesting points in the comparison of the analyses of these two hens is the great difference in the percentage of ash, while there is practically no difference in the percentage of calcium, magnesium, and phosphorus in the ash. In other words, there seems to be a stable equilibrium in this connection which exists even though there is a marked difference in the total quantity. As will be seen in Table VI, magnesium fails to adhere to this rule.

In Table VI the comparative analyses of the carcasses and leg bones of hens from Lots 1, 2, 3, and 4 show that the hens of Lots 1 and 2 weigh appreciably less than those of Lots 3 and 4 and that there is a general depletion of the quantities of ash, calcium, magnesium, and phosphorus in Lots 1 and 2.

It would seem from a study of the foregoing figures that the following conclusions are justified.

1. Laying hens whose supply of calcium is limited to that naturally occurring in the food will continue laying eggs until there is a general depletion of magnesium, phosphorus, and calcium in their bones and carcasses.

² McCrudden, F. H., The quantitative separation of calcium and magnesium in the presence of phosphates and small amounts of iron devised especially for the analysis of foods, urine and feces, *J. Biol. Chem.*, 1909-10, vii, 83.

2. As long as the economy of the hens permitted the formation of an egg shell, the contents of the egg remained reasonably constant, thereby permitting an average supply of calcium, magnesium, and phosphorus for the proper development of the embryo of the chick.

3. 10 weeks elapse before there is a noticeable change in the general condition of hens receiving no mineral matter other than that naturally occurring in the food such as that fed to Lots 1 and 2.

4. It would seem most probable that the breaking down of certain hens before the expiration of 6 months was caused by individual weakness.

5. Since no shell-less eggs were laid in Lots 1 and 2, it would indicate that the lack of calcium is not the fundamental cause of their formation.

6. The percentages of calcium and of phosphorus in the bones of the hens in all lots were reasonably constant during this experiment, thus indicating a stable equilibrium between the two elements.

7. The continued laying of eggs, under the calcium restrictions of this experiment, does not materially alter the percentage mineral composition of the egg shells or their contents. There is, however, a gradual thinning of the egg shells.

8. Under the conditions governing this experiment, the addition of limestone or oyster shell to the ration increases the production of eggs 69.4 per cent as shown by the average egg production of Lots 1 and 2 when compared with that of Lots 3 and 4.

NOTE ON A SHORT MODIFICATION OF THE OFFICIAL CHLORINE METHOD FOR FEEDS, FECES, AND URINE.

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The determination of chlorine in blood or body fluids has resulted in a modification of the Volhard method by Rappleye¹ and in the development of an iodometric method by McLean and Van Slyke² both of which aim to give better and more distinct end-points than the above official method³ and therefore more accurate results.

The concentration of the NaCl estimated by them in blood involved a magnitude of 400 to 600 mg. per 100 cc. of liquid. The magnitude of chlorine estimated in feeds, feces, and swine urine is less than 100 mg., consisting respectively of 39, 19, and 104 mg. or the actual amount estimated per determination was 3.8, 3.6, and 14.0 mg. The estimation of such small quantities by the official Volhard method as usually done does not give as consistent results as desired.⁴

This is chiefly because of the volumetric strength of the reagents employed, the indefiniteness of the end-point in titration, and the difficulty of washing out excess AgNO₃ from the precipitated AgCl without obtaining a slightly turbid filtrate which renders the end-point less distinct. The process of washing the excess AgNO₃ out is also long and tedious resulting in a bulky filtrate which gives a less distinct end-point.

¹ Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.

² McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

³ *J. Assn. Offic. Agric. Chem.*, 1915-16, i, p. xxxii.

⁴ Halverson, J. O., and Schulz, J. A., *J. Am. Chem. Soc.*, 1919, xli, 440.

For these reasons the modification given below was adopted. This consisted in eliminating the step of washing excess AgNO_3 out from the AgCl precipitate and substituting titration of an aliquot of the clear filtrate directly after filtering off the AgCl precipitate. This procedure gives the same results as the official method when the latter is refined by using weaker reagents of AgNO_3 and of NH_4CNS , and using a burette with the latter reading to 0.05 cc. in which the NH_4CNS is capable of being estimated to 0.01 cc. Further, a definite smaller volume of solution is always titrated.

The Proposed Modification.

After the digestion of the alkaline ash with dilute nitric acid the solution is filtered into a beaker and the residue washed with hot water to a total volume not exceeding 75 cc. To the boiling solution on the hot plate is gradually added 0.05 N AgNO_3 , chlorine being slowly precipitated. The volume is then reduced to 25 or 30 cc. by boiling which allows the precipitate to coagulate and gives a perfectly clear solution. The solution is allowed to cool, when it is carefully transferred to a 100 cc. volumetric flask, made up to mark, and is then gently mixed by inverting the flask several times. Let stand 3 to 4 hours or, if convenient, over night thus allowing the precipitate to settle. Filter through a dry filter paper into a dry beaker. Pipette out 95 cc. into a 200 cc. Erlenmeyer flask.⁵ Add 2 cc. of ferric nitrate indicator,⁶ titrate the excess AgNO_3 with NH_4CNS , which is one-half the strength of the AgNO_3 ,⁷ against a white background using a burette reading to 0.05 cc. and capable of being estimated to 0.01 cc.

The number of cc. of NH_4CNS used, increased by $\frac{100}{95}$, gives the equivalent cc. for the total charge taken. Cc. of AgNO_3 —
(cc. of $\text{NH}_4\text{CNS} \times \frac{100}{95} \times \text{factor}$) = cc. of AgNO_3 used.

⁵ A 47.5 cc. pipette is conveniently used here.

⁶ 125 cc. of concentrated nitric acid are added to 325 cc. of water; boiled until colorless. 50 cc. of a saturated solution of ferric nitrate are added.

⁷ 0.05 N AgNO_3 is used; 1 cc. is approximately equivalent to 1.5 mg. of chlorine.

In Table I are given results by the refined official Volhard method and also with the proposed modification included. It is seen that the small differences, part of a milligram, per 100 gm. or per 100 cc. are of the same order of magnitude as obtained by McLean and Van Slyke.²

TABLE I.
*Comparison of the Modified Chlorine Method with the Official Chlorine (Volhard) Method.**

Material tested.	Quantity taken.	Chlorine by official method.†		Quantity taken.	Chlorine by modified method.		Difference per 100 gm. or cc.
		Found.	Per 100 gm. or cc.		Found.	Per 100 gm. or cc.	
		mg.	mg.		mg.	mg.	mg.
Corn.....	10.00 gm.	3.99	39.9	10.00 gm.	4.08	40.8	0.9
Linseed oil meal.....	10.00 "	3.82	38.2	10.00 "	3.89	38.9	0.7
Wheat middlings.....	10.00 "	3.61	36.1	10.00 "	3.69	36.9	0.8
Swine feces 1.....	18.00 "	3.84	21.3	16.97 "	3.65	21.5	0.2
" " 2.....	18.09 "	3.24	17.9	18.31 "	3.47	18.9	1.0
" " 3.....	17.95 "	3.06	17.0	17.64 "	3.09	17.5	0.5
" " 4.....	18.04 "	3.74	20.7	18.61 "	3.96	21.3	0.4
" urine 5.....	10.00 cc.	9.49	94.9	10.00 cc.	9.43	94.3	0.6
" " 6.....	10.00 "	9.13	91.3	10.00 "	9.22	92.2	0.9
" " 7.....	10.00 "	12.10	121.0	10.00 "	12.18	121.8	0.8
" " 8.....	10.00 "	10.70	107.0	10.00 "	10.74	107.4	0.4

* All samples were done in triplicate. Averages are given.

† 1 cc. of AgNO_3 was equivalent to 1.492 mg. of chlorine while the ratio for NH_4CNS was 1.2755; the ratio of NH_4CNS for the modified method was 0.5076.

Both the official Volhard and the modified method were checked against salts of known chlorine content, also against dried c. p. NaCl . The proposed method varied from a few hundredths to 0.6 or 0.7 per cent while the official Volhard method by the usual procedure varied 1 to 2 per cent from theory.

By this method clear solutions of small definite volumes for titration are obtained thus avoiding the frequent turbidity encountered and the tedious washing of the AgCl precipitate free from the excess AgNO_3 .

The authors wish to thank Dr. E. B. Forbes for courtesies rendered.

A COMPARATIVE STUDY OF HEMOGLOBIN DETERMINATION BY VARIOUS METHODS.

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(Received for publication, November 3, 1919.)

The regeneration of hemoglobin and red cells following simple anemia has been studied in this laboratory for more than 2 years. A preliminary report of this work by Whipple and Hooper (13) has appeared and it is obvious that this curve of hemoglobin regeneration can be influenced by a number of diet factors. In this work it is essential that there be an accurate determination of hemoglobin. For this reason a comparative study of many hemoglobin methods was undertaken. It is believed that the method finally adopted for this work will be of value to other workers in the experimental field as well as to hospital and school laboratories where routine hemoglobin readings are so frequent. It need not be stated that much of the work expended upon routine hemoglobin determination is a total loss because the instrument used has not been standardized or the method is inaccurate. Too little is known as to the normal hemoglobin value in human beings as affected by age, altitude, climate, etc. No comparison is possible until some accurate standard is adopted for the routine work.

The earlier anemia work in this laboratory was done with Sahli's method using his modification of Gowers' instrument. It was soon apparent that incorrect results were being obtained and that the hemoglobin percentages were considerably higher than they should be when correlated with the other blood findings. The standard color tubes when checked against the oxygen capacity method of Van Slyke (12) showed great variations in color density. New tubes were purchased and when standardized showed much fading varying from 5 to 20 per cent. The

results obtained with these tubes were therefore sufficiently erroneous to warrant the discarding of this method for hemoglobin determination.

The Palmer carbon monoxide method was next tested and accurate results were obtained, provided the standard solutions were frequently checked.

Since the publication of Newcomer's method based on spectrophotometric data this method has also been carefully investigated, as well as a combination of Palmer's and Sahli's method with slight modifications described below.

History of Methods.

I. Acid Hematin Method.—Sahli (10), finding that methods employing artificial color standards were not satisfactory, brought forth the acid hematin method. Hemoglobin is converted into acid hematin by the addition of 0.1 N HCl and then compared with a standard of like material. Numerous criticisms of the method have appeared and as many modifications have been offered. Berczeller (2) claimed that the presence of lipoids alters readings. Stäubli (11) called attention to the time factor for maximum color development. Palmer (9) claimed that the standard is not permanent, that there is considerable delay in maximum color development, and that the method is not applicable for blood of different species. Haessler and Newcomer (4) offered a modification in the instrument used, using eleven standard tubes of different concentrations for comparison. Lilliendahl-Petersen (7) employed Sahli's principle in a Tallquist form. Newcomer (8) recently published a method of hemoglobin determination by comparing an acid hematin suspension of blood with a piece of brown-colored glass of definite thickness. The method is based on spectrophotometric data. The comparison is made with a Duboscq type of colorimeter.

II. Carbon Monoxide Method.—Hoppe-Seyler in 1892 (6) published his procedure of accurately determining hemoglobin in the form of carbon monoxide hemoglobin. The technical difficulties involved were too numerous for general adoption of the method.

Haldane (5) 8 years later revived Hoppe-Seyler's principle of hemoglobin determination but in a much simpler form. He used Gowers' instrument.

Palmer (9) in 1918 published a method which has found much favor. The principle is that of Hoppe-Seyler's procedure; i.e., a color comparison of carbon monoxide hemoglobin solution with a standard of known hemoglobin content. Ammonia solution is used as a diluent instead of water. The color comparison is made in a Duboscq colorimeter.

EXPERIMENTAL.

I. Palmer Method.—We have used Palmer's method with only slight modifications. All experiments carried on during this investigation have been done on dogs. All blood is obtained by venous puncture. About 10 cc. of blood are drawn from the jugular vein with a glass syringe and emptied into a graduated centrifuge tube containing 2 cc. of a 1.6 per cent sodium oxalate solution. The plasma obtained by centrifugalization is carefully pipetted off, the tube slightly tilted, and a 1 cc. calibrated pipette of small lumen quickly inserted with the finger closing the opening at the upper end. Blood is slowly drawn up to the 1 cc. mark, the pipette is thoroughly wiped on the outside, and its contents are transferred into a small test-tube. The pipette is carefully rinsed in 2 cc. of N salt solution previously measured with this same pipette and emptied into a test-tube similar to the one containing the blood. This salt solution, now containing some red cells, is then carefully added to the 1 cc. of blood and the whole thoroughly mixed avoiding of course too vigorous shaking. Extreme care must be taken with this procedure so that the suspension of packed red blood cells is truly a dilution of one in three. The latter was ascertained to be the most convenient dilution, for the hemoglobin of our normal dogs is usually considerably over 100 per cent, frequently showing readings of 130 to 140 per cent. From the diluted blood cells suspension a 1 per cent solution of blood is made. 1 cc. of the diluted blood, 1:3, is drawn up into the same pipette used for diluting the packed cells as well as for measuring the original amount, is transferred to a 100 cc. volumetric flask containing

the 0.4 per cent ammonia solution, and made up to the 100 cc. mark with this same diluent. The solution is thoroughly mixed and at once saturated with carbon monoxide and read immediately. The percentage of hemoglobin obtained is multiplied by three—the packed red cells having been previously diluted 1:3—and this figure again multiplied by the red cell percentage of the blood computed from the hematocrit readings. In order to determine the accuracy of this apparently roundabout procedure, which is employed to prevent further bleeding for hemoglobin determination, readings were made from whole blood collected from the vein directly into a vessel containing sufficient dry sodium oxalate to prevent clotting and compared with the figures

TABLE I.*

Hemoglobin of packed red blood cells diluted 1:3.	Undiluted packed red cells.	Red cells from hematocrit.	Estimated hemoglobin from packed cells.	Hemoglobin of whole blood direct.	Difference.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
69	207	46.0	95	94	+1
57	171	48.0	82	83	-1
67	201	40.0	80	80	0
59	177	47.2	84	84	0
62.1	186.3	50.5	94	94	0
76.9	230.7	42.6	98	97	+1
63.0	189	38.4	73	73	0

* This work has been repeated and similar results have been obtained.

obtained by our means. Table I demonstrates the accuracy of the method as we employ it. It is obvious that by increasing the amount of hemoglobin used we tend to diminish any error of the method. 1 cc. of packed red cells should give more accurate readings than 20 mm. of whole blood.

Palmer's technique for the preparation of his standards has been closely adhered to. Palmer used either defibrinated ox or human blood. We have tried whole as well as defibrinated blood. Furthermore, blood obtained from different species has been investigated. In some cases blood has been obtained aseptically and the standard prepared from it with all aseptic precautions. With other standards only the usual cleanliness has been exercised.

The figures in Table II demonstrate the amount of fading of standards during the time of observation.

TABLE II.
Change in Color Value of Palmer Standards of Hemoglobin.

Standard No.	Source of blood.	Aug. 8, 1918.	Aug. 23, 1918.	Sept. 20, 1918.	Oct. 21, 1918.	Nov. 15, 1918.	Dec. 24, 1918.	Jan. 24, 1919.	Mar. 5, 1919.	April 1, 1919.	May 16, 1919.	June 23, 1919.	Aug. 5, 1919.	Sept. 7, 1919.	Oct. 21, 1919.	Total fading. per cent	Remarks.
I	Dog (whole).	100	99	90	75	73	74	74	72	71						29	Discarded. brownish tinge last 3 months.
II	Goat " sterile.		100	85	80	73	74	74	75	75	76	75	75	75		26	Discarded. brownish tinge last 3 months.
III	Dog (defibrinated).			100	99	89	87	86	86	80	80	81	82	82	82	20	No color change.
IV	Human "					100	93	89	89	84	84	84	85	85	84	16	" "
V	Sheep "							100	98	91	91	92	90	90	90	10	" "
VI	Dog (whole).								100	93	88	87	85	86		15	Spoiled.
VII	" (defibrinated).									100	100	100	100	100	100	0	No color change.
VIII	" "										100	99	99	96	95	5	" "

Discussion of Table II.

No. I showed the greatest fading, 29 per cent in 8 months, the maximum change taking place during the first 2 months. The slight color change did not interfere with color comparison.

No. II was prepared from goat blood with aseptic precautions and kept sterile throughout the experiment. Neither the fragility of red cells nor bacterial decomposition seems to play an important rôle in the fading of color. The change was nearly as much as with No. I, the maximum fading taking place during the first 3 months.

The curve of No. III is more promising, defibrinated blood showing slightly better keeping qualities than whole blood. The fading was not so rapid nor so much as in Standards I and II, nor was any color change apparent.

No. IV prepared from pooled defibrinated human blood showed about the same stability of color as defibrinated dog blood. The greatest change took place during the first 2 months; after that the fading was slight.

We were more successful in keeping No. V, prepared from defibrinated sheep blood, for the maximum fading was but 10 per cent as compared with 16 and 20 per cent when using defibrinated human and dog blood.

No. VI, having as its source whole dog blood, faded 15 per cent during 6 months, not so much as Standards I and II also prepared from whole blood, but still slightly more than when the blood was previously defibrinated.

Standard VII again prepared from defibrinated dog blood was the only one which remained unchanged during the period of observation, 7 months.

Standard VIII, for which defibrinated dog blood was again employed, showed a fading of 5 per cent during 5 months. While more promising than the earlier ones, still the change is too much for accurate hemoglobin determinations.

Another standard prepared from whole dog blood to determine again the difference obtained with whole and defibrinated blood is but 2 months old. The fading already amounts to 7 per cent during this period.

II. Acid Hematin Method.—Because of the uncertain stability of carbon monoxide hemoglobin solutions we have attempted to solve the problem by searching for a more stable color standard. In view of Sahli's work acid hematin was tried again. It offers an easier color comparison than do the reds of oxyhemoglobin and carbon monoxide hemoglobin. Sahli in his original work obtained satisfactory results, as did several of his coworkers. We have combined Palmer's method with Sahli's principle, that is, determined hemoglobin in the form of acid hematin, employing Palmer's method of standardization. The sealed tubes containing dilute acid hematin suspension as purchased (Sahli instrument) are unsatisfactory as was pointed out in the history of the methods. The thought that acid hematin in a more concentrated form might not fade so readily arose, and therefore 5, 10, and 20 per cent suspensions were investigated. Our method of procedure was as follows:

The oxygen capacity of a sample of dog's blood was determined by Van Slyke's method and the hemoglobin content computed therefrom. An acid hematin standard in the form of a 20 per cent suspension, so diluted that a 1 per cent dilution prepared from it would read 100 per cent, was made. Because of our experience with whole and defibrinated blood in the preparation of standards for the carbon monoxide method we employed defibrinated blood for our first standard. It is well known and has been mentioned in the history of acid hematin methods that the time factor for allowing the maximum color of acid hematin to develop plays a very important rôle. This standard, after preparation and dilution, was allowed to stand 24 hours to insure correct readings. A 1 per cent dilution prepared from the 20 per cent suspension was of course employed for direct color comparison. The same time factor was used for the blood to be tested. A 1 per cent suspension of blood is used for the determination of hemoglobin. 0.1 *N* HCl is employed throughout the procedure, for the original standard suspensions as well as for all further dilutions. The strength of HCl used within a certain limit is immaterial. We tried *N*, 0.1 *N*, and 0.5 *N* and obtained identical readings. Stronger solutions than *N* caused precipitation. The entire amount of diluent is used at once, that is, 1 cc. of diluted packed red cells is discharged

into the volumetric flask containing about 50 cc. of 0.1 N HCl, then mixed, and the volume made up to the 100 cc. mark with 0.1 N HCl. The suspension is then allowed to stand 24 hours in the ice chest, thoroughly shaken again, for it must be remem-

TABLE III.
Hemoglobin.

Palmer's method.	Author's modification.
<i>per cent</i>	<i>per cent</i>
75	74
70	70
77	76
77	77
74	73
73	73
74	74
75	74
76	75
73	73
64	64
63	63
76	76
45	45
60	60
84	85
69	69

TABLE IV.

Van Slyke's oxygen capacity method.	Palmer's method.	Author's modification.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
113	113	114
117	118	117
99	98	99
124	124	124
109	108	109

bered that we are dealing with a suspension and not a solution, and then read. The method of hemoglobin determination is exactly like that of Palmer's method and the readings obtained are almost identical as illustrated by the figures given in Tables III and IV.

The source of light used for color comparison makes little difference. Light from a northern exposure or that originating from a nitrogen-filled bulb filtered through "Daylite" glass gives equally good readings. The slight turbidity of the acid hematin suspension, and it is very slight in a 1 per cent dilution, does not in the least interfere with an accurate color comparison.

Different standards have been prepared, 5, 10, and 20 per cent suspensions, defibrinated as well as whole blood, also blood from different species. In fact, standards have been made up from the same sample of blood, one diluted for carbon monoxide hemoglobin determinations and the other in the form of acid hematin suspensions. The standards have been checked up once a month as those for Palmer's method. Material for the 1 per cent dilution has been withdrawn from the stock bottles once each week, and the container resealed with paraffin. On prolonged standing some of the hematin settles to the bottom of the container but when the mixture is thoroughly shaken again the readings obtained are unchanged. It is of course very essential that all acid hematin suspensions, whether dilute or concentrated, are thoroughly mixed before using.

The keeping qualities, or rather stability of color density, are best demonstrated by Tables V, VI, VII, and VIII.

Standard IVa (Table V) faded 4 per cent during a time interval of 11 months. The change was apparent during the 1st month. In the same period of time the carbon monoxide standard had faded 16 per cent.

These tables (V, VI, VII, and VIII) demonstrate the keeping qualities of our acid hematin mixtures. Up to the present time the last three have remained practically unchanged. We shall continue to check up these suspensions once each month in order to determine just how long they will remain stable.

A 1 per cent standard diluted from Standard VIIa at the time of preparation, April 1, 1919, and simply kept in an Erlenmeyer flask in the ice chest still read 100 per cent on October 21, 1919. Another 1 per cent suspension diluted from the defibrinated sheep blood standard and kept under the same conditions as the one mentioned above also remained unchanged during the period of observation, 4 months. For exactly how long a period the 1 per cent dilution would remain stable we do not at present

know. We consider it safe to make up our 1 per cent suspensions from the stock mixture once each month. It may be of some importance that all these standards were preserved in an

TABLE V.

Standard IVa; Acid Hematin. Defibrinated Human Blood Prepared Nov. 25, 1918.

Date.	New standard prepared.	Standard IVa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.	
1918						
			per cent	per cent		
Dec. 24.....	100	97	3	7	Carbon monoxide standard was prepared from the same sam- ple of blood.	
1919						
Jan. 24.....	100	97	3	11		
Mar. 5.....	100	96	4	11		
Apr. 1.....	100	97	3	16		
May 15.....	100	96	4	16		
June 23.....	100	97	3	16		
Aug. 6.....	100	96	4	15		
Sept. 7.....	100	96	4	15		
Oct. 21.....	100	96	4	16		

TABLE VI.

Standard Va; Acid Hematin. Defibrinated Sheep Blood Prepared Jan. 24, 1919.

Date.	New standard prepared.	Standard Va.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
<i>1919</i>					
			<i>per cent</i>	<i>per cent</i>	
Mar. 5.....	100	100	0	2	Carbon monoxide standard was prepared from the same sample of blood.
Apr. 1.....	100	100	0	9	
May 15.....	100	100	0	9	
June 23.....	100	100	0	8	
Aug. 6.....	100	100	0	10	
Sept. 7.....	100	100	0	10	
Oct. 21.....	100	100	0	10	

ice chest with fairly constant temperature, fluctuations rarely exceeding 1-4°C.

TABLE VII.

Standard VIa; Acid Hematin. Whole Dog's Blood Prepared Mar. 5, 1919.

Date.	New standard prepared.	Standard VIa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
1919			per cent	per cent	
Apr. 1.....	100	100	0	7	Carbon monoxide standard prepared from the same sample of blood.
May 15.....	100	100	0	12	
June 23.....	100	99	1	13	
Aug. 6.....	100	100	0	15	
Sept. 7.....	100	100	0	14	
Oct. 21.....	100	100	0	Spoiled.	

TABLE VIII.

Standard VIIa; Acid Hematin. Defibrinated Dog's Blood Prepared Apr. 1, 1919.

Date.	New standard prepared.	Standard VIIa.	Amount of fading.	Amount of fading of carbon monoxide standard.	Remarks.
1919			per cent	per cent	
May 15.....	100	100	0	0	Carbon monoxide standard prepared from the same sample of blood.
June 23.....	100	100	0	0	
Aug. 6.....	100	100	0	0	
Sept. 7.....	100	100	0	0	
Oct. 21.....	100	100	0	0	

III. Newcomer Method.—While this investigation was being carried on Newcomer published his new method of estimation of hemoglobin, details of which have been described above. A piece of this brown semaphore glass 0.96 mm. in thickness was tested and gave most satisfactory readings when compared with Palmer's figures as shown in Table IX.

A Duboscq colorimeter is used in this laboratory and the brown glass inserted above the plunger. The corresponding cup is partially filled with distilled water for the reasons mentioned by Newcomer. As a source of light for these hemoglobin determinations either the lamp containing "Daylite" glass or light from a northern exposure gave equally satisfactory results. While the colors of the glass 0.96 mm. in thickness and the acid hematin suspension of the blood to be tested matched satisfac-

torily, the color is very light, almost a lemon-yellow when matched. It is therefore quite evident that this is somewhat of a disadvantage for darker shades are more easily matched and of course there is no means of regulating the depth of standard color as one does when using liquid mixtures. For example, with our liquid standards set at 10 the resulting readings of the test fluids range usually between 10 and 13, while with the colored glass, at least with the piece of this particular thickness, the readings are around 5 to 7 and are not so accurate as those around 10 or 12 with the Duboscq instrument.

TABLE IX.

Palmer's method.	Newcomer's method (glass 0.96 mm. thick).	Difference.
<i>per cent</i>	<i>per cent</i>	
113	114	+1
116	114	-2
99	100	+1
107	108	+1
98	101	+3
117	118	+1
100	100	0
100	100	0
100	101	+1
100	103	+3
100	99.2	-0.8
Average difference		+0.73

The added advantage of course is that all artificial standards like this glass are supposedly permanent in color and this obviates the necessity of liquid standard preparations. Because of the pale color of the standard glass we purchased another piece somewhat thicker—1.02 mm.—hoping to obtain easier readings. While the color was slightly darker in this new piece our readings were not so accurate as is evident from the figures cited (Table X).

The difference in readings (Table X) was more than with the piece 0.96 mm. in thickness, an average of 0.73 higher with the former glass as compared to 2.4 points lower with the thicker one. We feel certain that this larger difference is due to the

fact that with the piece 1.02 mm. thick the color, although darker, is not so readily matched. The suspension of acid hematin demonstrates of course a very slight turbidity, the lack of which is very noticeable when using the heavier glass. The color of the latter is a clear yellowish brown while the acid hematin because of its slight opaqueness seems a somewhat different shade of brown. Some of the readings cited were made by different workers in the laboratory and the same difficulty was voiced by all that the colors do not seem to be quite the same. Two pieces of glass placed one on top of the other, each 1.02 mm. in thickness, increased the difference in readings considerably, as

TABLE X.

Palmer's method.	Newcomer's method (glass 1.02 mm. thick).	Difference.
<i>per cent</i>	<i>per cent</i>	
64	62	-2
63	60	-3
76	72	-4
45	47	+2
60	56	-4
84	81	-3
69	66	-3
Average difference		-2.4

well as the difficulty of exact color match. Newcomer (8) states that it is impossible to secure an artificial color match which runs true through a range of thicknesses. While the actual difference in thickness of the pieces of glass purchased—using of course only one at a time—seems slight, a decided difference in color match is apparent.

In view of the above mentioned difficulties we prefer using the liquid acid hematin standards.

DISCUSSION.

In summarizing our observations with the Palmer method it is evident that as long as the standard solutions are prepared once a month very accurate results may be obtained. The method itself is certainly simple and easily carried out by even

comparatively inexperienced laboratory workers. The main disadvantage lies in the color fading of the standard solutions. Among nine different standards observed for a period of from 2 to 13 months we have found but one solution which for nearly 7 months remained unchanged. The remaining eight all faded sufficiently to prohibit their use for accurate work, with one exception possibly—Standard VIII—which demonstrated a fading of only 5 per cent during a 5 months period. Considering the figures presented it seems that better results were obtainable with blood previously defibrinated than with whole blood. For a time interval of 3 months the standard solutions prepared from defibrinated blood demonstrated a fading of from 0 to 13 per cent as compared with 13 to 27 per cent evident in those mixtures originating from whole blood. We have never encountered any difficulty with reference to a true change of color, at least during a period of 6 months. During the last few months of observation a very slight brownish tinge was noticeable but never sufficient to interfere seriously with a color comparison, excepting perhaps Nos. I and VI, which were discarded after 8 months observation. The greatest change in color density in standards prepared from whole blood apparently takes place during the first 2 months and reaches its maximum during the 3rd month. From then on the change is but comparatively little and remains so, in some instances for a year or over. With the defibrinated blood, I think we have a standard of better keeping qualities; the change is not quite so pronounced. Although two of the defibrinated blood mixtures, Nos. III and IV, showed a fading of 11 per cent each during a time interval of 2 months, we have three others where the change was considerably less, one solution demonstrating but a 5 per cent loss of color within 5 months, another remaining unchanged for nearly 7 months, and a third fading 10 per cent in 9 months.

The type of hemoglobin evidently plays no important rôle, dog's blood apparently giving as satisfactory results as goat's, sheep's, or human blood; or rather the other species mentioned offer no more stable hemoglobin solutions than dog's blood.

Bacterial decomposition does not seem to be a very important factor, for blood obtained aseptically and the hemoglobin solution prepared with sterile precautions demonstrated no more

stable qualities than did those standards prepared with only the usual care and cleanliness.

It should be mentioned here that all these stated observations pertain to the stock solutions, the 20 per cent dilutions. We have never attempted to keep the 1 per cent dilutions prepared from the concentrated mixtures for more than a week, at least under experimental conditions existing here. A 1 per cent solution prepared will not fade within a week if kept on ice and resaturated with carbon monoxide each time the container is opened. After that time interval a change does take place which although not apparent to the eye is readily demonstrable when checked against the oxygen capacity method. Appleton (1) states that the 1 per cent solutions prepared by her began to deteriorate in from 2 to 4 weeks. During her investigation the diluted solution was kept saturated by a continual flow of gas. Why such a difference in stability occurs we do not know. Frequent opening of containers and resaturation with CO seem to give no better results than resaturation once a month. While a standard is being used for routine work it is necessary to open the container once each week in order to procure the necessary material for one 1 per cent dilution.

From the tables of acid hematin standards, it is readily seen that, while these acid hematin mixtures may change in time, they certainly have proved themselves to be much more stable than the carbon monoxide solutions. One may with perfect safety and with complete assurance of obtaining accurate results employ these acid hematin suspensions for 6 months at least. The stock mixtures above mentioned will of course be observed to determine just how long they actually remain unchanged.

The method of preparing blood for hemoglobin determinations in the form of acid hematin is slightly simpler than the carbon monoxide method, for the former makes unnecessary the extra step of saturation with carbon monoxide. The slight disadvantage is the time interval necessary for the maximum color of acid hematin to develop. In using large quantities of blood (at least much greater amounts than are used clinically) we have allowed 24 hours. This, however, is not necessary as a 1 hour interval gives accurate reading. We have observed no difference between figures obtained after 60 minutes standing and 24 hours.

The latter time happened to be more convenient in our experimental work. Newcomer published a table with his method showing the exact percentages of color development of acid hematin in given periods of time. He considers 40 minutes as safe.

Palmer in his publication states that blood of different species cannot be used for hemoglobin determinations in the form of acid hematin. As will be seen from our tables, we have standards prepared from human, dog's, and sheep's blood and have compared human blood with both dog's and sheep's blood standards without encountering any difficulty whatsoever. The comparisons have been made in all combinations possible with our material and readings have checked accurately. The figures presented in the tables readily answer the question of stability. While the latter may not be permanent or remain unchanged indefinitely, still it is much less time-consuming to prepare a fresh standard once every 6 months instead of once each month as we have had to do when employing the carbon monoxide method.

The Newcomer method would of course be the best solution of the entire problem, but as mentioned before the color match is not exact. The use of as simple a standard as a piece of colored glass certainly is a great advantage. The difference in color may not be so apparent to all eyes. The table accompanying the glass standard is an asset, as it definitely settles the question of time interval for development of the maximum color of acid hematin, and thus does away with one of the disadvantages of the earlier acid hematin methods.

Since the completion of this work a communication of Cohen and Smith (3) has appeared which confirms much of this work. They suggest the same standard solution because of its stability under army camp conditions.

CONCLUSIONS.

1. The Sahli hemoglobin method when using the color tubes accompanying the instrument gives very inaccurate results because of the decided variance in color density of the standard tubes, due to fading.

2. The Palmer method offers very satisfactory means of hemoglobin determinations if the standard solutions are freshly prepared. The method itself is very simple and may be successfully carried out by anyone familiar with colorimetry. The standard solutions prepared in the laboratory although carefully made have not been sufficiently stable to insure accurate determinations over periods of more than 3 to 4 weeks.

3. Newcomer's method obviates many difficulties heretofore observed with other procedures and gives good results with the glass 0.96 mm. in thickness, although the color is quite pale. When using the heavier glass, 1.02 mm. in thickness, the color match is only approximate and the figures obtained are not so satisfactory as those resulting from use of the thinner piece.

4. A method is presented applying Palmer's procedure to Sahli's principle which has proved most satisfactory. It removes the difficulty we encountered with Palmer's method, the lack of stability of color in the standard solutions. It has the advantage of an easier color match than that of red tint. The standards prepared have remained sufficiently unchanged for a period of 11 months to insure accurate hemoglobin determinations during this long period.

It may be suggested that for routine hospital work an acid hematin standard prepared in this way and kept at relatively constant temperature will remain unchanged for 8 months or longer. 1 per cent solutions may be prepared from time to time from the standard concentrated solution and this 1 per cent solution can be used to fill the standard tube of the common Sahli hemoglobinometer. This insures an accurate base line for hemoglobin determinations and with refilling of the Sahli tubes once a month will give accurate clinical determinations. Such clinical determinations are not the rule and are much to be desired.

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DIGESTIBILITY OF CERTAIN MISCELLANEOUS VEGETABLE FATS.*

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INTRODUCTION.

The shortage of food fats which existed during the war will, in all probability, be more or less permanent. For this reason it seemed desirable to obtain information on the nutritive value of some oils which have either not been used as foods or only used as such to a limited extent, but which might perhaps be thus used in case of need.

This office has made similar studies of the digestibility of about 50 more or less common fats and oils (1). The results seem to indicate that the digestibility coefficient of the fats having melting points above body temperature (37°) varies inversely with the melting point (2). It has also been found that most of the common oils are from 93 to 98 per cent utilized by the human body. Nevertheless, some oils were found to cause digestive disturbances and some oils are less tolerated by the body than others. Therefore, before we can say whether or not an oil or fat is suitable for use in the human dietary, we must know not only its coefficient of digestibility but also its effect on the human body when eaten in quantities at least equal to those in which butter or other common fat is used in the average dietary.

This paper records the digestibility of avocado and cupuassú bean fats, and cohune, hempseed, palm-kernel, and poppy-seed oils. Avocado fat is eaten largely in parts of the tropics, and

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to some extent in this country as it occurs in the fruit; but the other oils studied have been used for food purposes only in a limited way, if at all, in this country. The study of the nutritive value of these oils seemed valuable not only on account of its interest in the field of nutrition but also on account of its very practical bearing upon our food supply, especially in times of shortage of the more common fats.

EXPERIMENTAL.

General Procedure.

The methods followed in the tests here reported are essentially the same as those followed in the previous digestion experiments conducted by this office. The fats, with the exception of avocado fat, were incorporated in a special corn-starch blanc-mange or pudding, and this was eaten with a basal ration consisting of a commercial wheat biscuit, oranges, and sugar, which supplied only a very small amount of fat in the total ration in comparison to that eaten in the blanc-mange. The avocado fat was eaten as it occurs in the fresh fruit pulp with a basal ration of milk and crackers, which supplied considerably less than one-half as much fat as was furnished by the avocados. Tea or coffee without cream was used if desired.

The amount of food eaten was accurately weighed and the weight of the water-dried feces for the experimental period was recorded. The methods for the separation of feces, analyses, etc. were the same as those reported in earlier papers (1).

The subjects who assisted in these tests were men, apparently in normal health, ranging in age from 20 to 40 years, who were students in local universities. They were familiar with this type of work and thoroughly trustworthy.

I. Avocado Fat.—The avocado (*Persea gratissima*) is a fruit indigenous to tropical and subtropical regions in the western hemisphere. In the United States it is cultivated to an increasing extent in Florida and California. The avocado, because of the large amount of fat it contains and because of its pleasant taste, seemed of interest to study. The avocados which we used in our experiments contained about 15 per cent fat, although Condit and Jaffa (3) state the average fat content to be about 20 per

cent. It is supposed to be present in the form of an emulsion, which probably accounts for the difficulty in expressing it from the pulp (Table I). Mattil (4), in two tests made during the progress of our experiments, using a basal ration of Graham crackers, cottage cheese, and milk, reports the digestibility of the total fat in a diet in which avocado fat was the predominating fat constituent as 93.7 per cent and 89.1 per cent. If the same correction which we made in our experiments for the fat of the diet of accessory foods is applied, the average of Mattil's figures becomes practically identical with the 88 per cent which we report for the digestibility of avocado fat alone. There were no

TABLE I.

Summary of Digestion Experiments with Avocado Fat in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of avocado fat alone.
		Protein.	Fat.	Carbo-hydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
527	H. R. G.	85.0	90.8	96.1	74.9	88.3
529	P. K.	88.0	90.4	97.4	76.0	88.5
530	C. J. W.	84.5	88.7	97.6	73.7	86.8
Average		85.8	90.0	97.0	74.9	87.9

physiological disturbances caused by the ingestion of the avocado fat. The maximum intake by one subject was 124 gm. of avocado fat per day, while the average intake per man per day was 100 gm.

II. Cohune Oil.—Cohune oil is obtained from the kernel of the cohune palm (*Attalea cohune*), which grows quite extensively in Central America. The oil resembles coconut oil both in taste and odor, but has not been used very largely, due to the great difficulty experienced in breaking the very hard outer shell of the nut. At room temperature, the oil¹ has only a trace of solid fat. The subjects ate on an average of 52 gm. of cohune oil per man per day, while one subject ate 64 gm. per day for the experimental period (Table II). The oil was very well assimilated, being on an average 99 per cent digested, and produced no abnormal physiological effects.

¹ The sample used in this experiment was obtained through the courtesy of H. S. Bailey of the Bureau of Chemistry.

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III. Cupuassú Fat.—The cupuassú fat is obtained by pressing the seeds which occur in the pulp of the fruit of the cupuassú tree (*Theobroma grandiflora* Schum), which is one of the most important fruit trees of Pará, Brazil. Cupuassú fat has not been used for food purposes in this country. Nevertheless it seemed

TABLE II.

Summary of Digestion Experiments with Cohune Oil in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of cohune oil alone.
		Protein.	Fat.	Carbo-hydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
621	A. A. F.	64.0	94.7	96.2	66.2	99.5
622	P. K.	68.8	94.9	97.0	80.0	98.6
623	J. C. M.	53.8	94.0	94.5	69.5	99.3
624	C. J. W.	67.3	94.6	95.6	77.5	98.8
Average		63.5	94.6	95.8	73.3	99.1

TABLE III.

Summary of Digestion Experiments with Cupuassú Fat in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of cupuassú fat alone.
		Protein.	Fat.	Carbo-hydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
637	A. A. F.	78.4	91.0	97.4	63.3	95.3
638	P. K.	69.1	88.4	96.5	48.8	94.1
639	J. C. M.	77.5	89.6	96.1	54.2	95.0
640	C. J. W.	75.7	86.3	96.2	57.0	92.1
Average		75.2	88.8	96.6	55.8	94.1

to be a fat worth attention, since on account of the abundance of the cupuassú trees in Brazil it would be available in fairly large quantities.

It is a light yellow fat, solid at ordinary temperatures, and possessing the odor of cacao butter (Table III). The average digestibility, 94.1 per cent, found for cupuassú fat agrees very closely with that of cocoa butter, 94.7 per cent found in previous investigations (5). Only 41 gm. of cupuassú fat were consumed

on an average per man per day. The cupuassú fat caused slight physiological disturbances, such as nausea and looseness of the bowels, similar to those noted in the experiments with cocoa butter.

IV. Hempseed Oil.—Hempseed oil is expressed from the seeds of the hemp plant (*Cannabis sativa*). It is a yellowish green oil which has had practically no use as a food in this country, but has been almost wholly used for industrial purposes. It was eaten without aversion and caused no physiological disturbances. In our experiments hempseed oil was very completely assimilated, being on an average 98.5 per cent digested (Table IV). The subjects ate on an average 54 gm. per man per day, while one subject ate 57 gm. per day without any ill effects.

TABLE IV.

Summary of Digestion Experiments with Hempseed Oil in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of hempseed oil alone.
		Protein.	Fat.	Carbo-hydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
906	J. F. C.	79.1	94.4	97.4	75.8	98.1
909	G. S. M.	56.4	94.7	96.3	65.0	99.5
910	W. O'C.	65.8	94.0	97.2	63.9	97.9
Average		67.1	94.4	97.0	68.2	98.5

V. Palm-Kernel Oil.—Palm-kernel oil is one of the two oils obtained from the fruit of an African palm tree (*Elaeisis guineensis*). It is obtained by pressing the interior portion of the kernels. Palm oil, obtained from the exterior of the fruit, is rarely used for food purposes outside of the countries in which it is produced, but finds commercial application in the United States in the tin plate industry. The palm-kernel oil used in this experiment was obtained by cracking the kernels by hand and expressing the oil from the meats by a small sized, continuous process oil expeller. The oil possessed a pleasant odor and flavor much resembling coconut oil, but had a somewhat higher melting point, being a white solid at ordinary temperatures. The subjects ate on an average 100 gm. of palm-kernel oil per man per day

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(Table V). The maximum intake was 121 gm. per day for the experimental period. The palm-kernel oil was very well assimilated, being 98 per cent utilized by the body, and it caused no physiological disturbances.

TABLE V.

Summary of Digestion Experiments with Palm-Kernel Oil in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of palm-kernel oil alone.
		Protein.	Fat.	Carbo-hydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
685	A. A. F.	56.3	94.2	96.1	32.6	97.7
686	P. K.	81.0	93.9	99.2	82.7	95.1
687	J. C. M.	67.2	97.5	96.5	64.8	99.9
688	A. A. R.	39.3	95.7	95.6	45.7	99.3
Average		61.0	95.3	96.9	56.5	98.0

TABLE VI.

Summary of Digestion Experiments with Poppy-Seed Oil in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of poppy-seed oil alone.
		Protein.	Fat.	Carbo-hydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
956	J. F. C.	62.4	92.8	96.1	57.3	96.1
957	F. K.	47.3	91.4	96.6	58.7	96.9
958	G. S. M.	67.0	93.4	96.7	56.7	98.5
959	W. O'C.	38.7	89.6	97.2	41.3	94.8
981	E. L. M.	58.0	89.2	96.4	47.7	95.1
982	G. S. M.	58.7	95.5	96.0	57.2	100.0
983	W. O'C.	11.5	87.4	96.5	32.7	92.9
Average		49.1	91.3	96.5	50.2	96.3

VI. Poppy-Seed Oil.—Poppy-seed oil is obtained by pressing the oil from the seeds of *Papaver album* and *Papaver nigrum*, two varieties of poppy, which are largely grown in the Orient. The best qualities of oil are used largely for edible purposes in Europe, but to a much smaller extent here. The best grades are used also for

artists' paints and other special technical purposes, while poorer qualities are used for soap-making. The oil used in these experiments was a good grade commercial oil which had a light yellow color and was without pronounced odor or flavor. It had the

TABLE VII.
Average Amount of Blanc-Mange and Total Food per Man per Day.

Diet.	Weight.	Water.	Protein.	Fat.	Carbohydrate.	Ash.	Fuel value.
	gm.	gm.	gm.	gm.	gm.	gm.	cal.
Avocado fat:							
Fat-rich avocado pulp . . .	688.4	547.8	6.0	99.8	30.0	4.7	1,042
Total food	1,603.4	1,194.9	43.0	153.9	198.2	13.5	2,350
Cohune oil:							
Fat-rich blanc-mange . . .	650.2	342.9	14.2	52.3	234.4	6.4	1,465
Total food	1,131.8	628.9	28.2	54.6	410.4	9.7	2,246
Cupuassú fat:							
Fat-rich blanc-mange . . .	536.0	310.9	22.5	39.9	159.5	3.2	1,087
Total food	902.6	496.5	36.3	42.0	321.6	6.1	1,810
Hempseed oil:							
Fat-rich blanc-mange . . .	406.8	177.2	7.3	53.5	165.7	3.1	1,174
Total food	1,026.2	538.5	21.2	55.8	403.9	6.7	2,205
Palm-kernel oil:							
Fat-rich blanc-mange . . .	577.7	251.9	9.8	100.3	213.0	2.7	1,785
Total food	1,001.2	490.7	22.9	102.4	379.5	5.6	2,531
Poppy-seed oil:							
Fat-rich blanc-mange . . .	356.8	154.9	6.4	49.0	144.5	2.3	1,045
Total food	916.3	521.6	17.8	50.9	320.4	5.6	1,810

following constants: Iodine number 135.50, refractive index at 40°C. 63, and 1.24 per cent of free fatty acid. The subjects ate on an average 50 gm. of poppy-seed oil and one subject ate 60 gm. daily for the experimental period. The oil caused no physiological disturbances and was well assimilated by the body, being 96.3 per cent digested (Table VI).

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Table VII reports the average amount of the fat-rich blanc-mange eaten per man per day and also the average total food consumed. Table VIII reports the coefficients of digestibility obtained by averaging the results of each group of experiments.

TABLE VIII.

Average Digestibility of Diet and Estimated Digestibility of Fats Studied.

Kind of fat.	No. of experiments.	Digestibility of entire ration.				Estimated digestibility of fats studied.
		Protein.	Fat.	Carbo-hydrates.	Ash.	
		per cent	per cent	per cent	per cent	per cent
Avocado fat.	3	85.8	90.0	97.0	74.9	87.9
Cohune oil.	4	63.5	94.6	95.8	73.3	99.1
Cupuassú fat.	4	75.2	88.8	96.6	55.8	94.1
Hempseed oil.	3	67.1	94.4	97.0	68.2	98.5
Palm-kernel oil.	4	61.0	95.3	96.9	56.5	98.0
Poppy-seed oil.	7	49.1	91.3	96.5	50.2	96.3

SUMMARY.

1. The digestibility of several fats and oils has been studied. The digestibility coefficients obtained were: Avocado fat, 87.9 per cent; cohune oil, 99.1 per cent; cupuassú fat, 94.1 per cent; hempseed oil, 98.5 per cent; palm-kernel oil, 98.0 per cent; and poppy-seed oil, 96.3 per cent.

2. The digestibility of the protein and carbohydrate of the entire ration was essentially the same as that in other experiments of a similar nature, indicating that the fats exercised no unusual effect on the utilization of these constituents.

3. These fats and oils, with the possible exception of cupuassú fat, which caused slight disturbances, produced no abnormal physiological effects and may be regarded as satisfactory for food purposes. Cohune, hempseed, palm-kernel, and poppy-seed oils especially are very highly utilized by the human body.

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A METHOD FOR THE DETERMINATION OF METHEMOGLOBIN IN BLOOD.

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(Received for publication, December 29, 1919.)

In the course of experimental work on the production of methemoglobin in pneumococcic infections it became desirable to have some simple method for the determination of methemoglobin in blood. The author was unable to find in the literature any method other than the complex and time-consuming spectrophotometric method, which requires an elaborate apparatus and is not very suitable when many determinations have to be made. The method outlined below is simple, quickly performed, and has given satisfactory results in a study of methemoglobin formation which will be published shortly.

Principle of the Method.—The method depends upon the fact that both hemoglobin and methemoglobin are changed quantitatively to cyanhemoglobin by dilute solutions of potassium cyanide. The color of the latter is a brilliant orange-red and is very suitable for colorimetric comparison. The change of methemoglobin to cyanhemoglobin is rapid, even in the cold. Hemoglobin, however, changes slowly at room temperature, and must be heated to 50°C. for about $\frac{1}{2}$ hour for complete conversion. That the cyanhemoglobin from hemoglobin is identical with that from methemoglobin is shown by the identical absorption spectra in the two cases.

In practice the difficulty arising from the slow conversion of hemoglobin into cyanhemoglobin is avoided by converting all the hemoglobin present into methemoglobin by the use of a little potassium ferricyanide, and then converting the methemoglobin into cyanhemoglobin. The resulting solution of cyanhemoglobin is compared with a standard of known strength in a Duboscq colorimeter.

The total amount of hemoglobin plus methemoglobin having been thus determined colorimetrically, the hemoglobin content of the blood containing the two pigments (hemoglobin and methemoglobin) is determined separately from the oxygen capacity, employing the technique outlined in Van Slyke's¹ gasometric determination of hemoglobin. The hemoglobin determined by the oxygen capacity is subtracted from the hemoglobin plus methemoglobin determined together as cyanhemoglobin; the difference is the methemoglobin.

Details of the Method.—Oxalated whole blood is used. 2.00 cc. of the blood are placed in a 100 cc. flask and 50 cc. of water are added which effect hemolysis in a few seconds. 0.5 cc. of a 0.1 M (3.0 per cent) solution of potassium ferricyanide is added, and the

TABLE I.
Factors for Calculating Results from Analysis of 2 Cc. of Blood Saturated with Air.

Temperature.	Air physically dissolved by 2 cc. of blood. Subtract from gas volume to obtain corrected gas volume representing O ₂ set free from hemoglobin.	Factor by which corrected gas volume is multiplied in order to give hemoglobin in 100 cc. of blood.
°C.	cc.	gm.
15	0.037	$34.7 \times \frac{B}{760}$
16	0.036	34.6 "
17	0.036	34.3 "
18	0.035	34.2 "
19	0.035	34.0 "
20	0.034	33.9 "
21	0.033	33.7 "
22	0.033	33.5 "
23	0.032	33.4 "
24	0.032	33.1 "
25	0.031	33.0 "
26	0.030	32.9 "
27	0.030	32.6 "
28	0.029	32.5 "
29	0.029	32.3 "
30	0.028	32.1 "

¹ Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.

flask allowed to stand for 15 to 20 minutes. (It was found that these conditions are optimum for the complete conversion of the hemoglobin to methemoglobin, only the faintest visible hemoglobin band being present at the end of 20 minutes with this amount of potassium ferricyanide.) 5 cc. of a 0.1 per cent potassium cyanide solution are now added. The change to cyanhemoglobin is immediate. Water is added to the mark and the solution compared with a standard of known strength in a colorimeter. The result is the hemoglobin plus methemoglobin, which we express as gm. of "total hemoglobin" per 100 cc. of blood.

A small portion (4 to 5 cc.) of the blood or hemoglobin solution is aerated in a funnel and its total oxygen capacity determined by the Van Slyke method. Barcroft² has shown that under these conditions (180 mm. of oxygen tension, 0 mm. of carbon dioxide tension, and room temperature) the hemoglobin is practically 100 per cent saturated. Therefore the oxygen capacity corresponds to the amount of hemoglobin present, and by dividing by 1.34 (the volume of oxygen combined with 1 gm. of hemoglobin) we obtain the gm. of hemoglobin per 100 cc. of blood. For convenience of calculation the factors for the conversion of cc. of gas combined with 2 cc. of blood into gm. of hemoglobin per 100 cc. of blood are given in Table I (modified from Van Slyke¹).

Preparation of Standard.—The standard is prepared from fresh whole oxalated or defibrinated blood which is known to contain no methemoglobin. The hemoglobin content (gm. per 100 cc.) is determined gasometrically. 500 cc. of standard are made by placing 10 cc. of the blood in a 500 cc. flask, hemolyzing with about 300 cc. of water, and adding 2.5 cc. of the potassium ferricyanide solution. After 20 minutes, 25 cc. of the potassium cyanide solution are added and the mixture is diluted to the mark. The blood pigment value of this solution is known from the gasometric determinations and the unknown may be compared directly with it or suitable dilutions of the standard may be made.

² Barcroft, J., *The respiratory function of the blood*, Cambridge, 1914.

Calculation of Results.—An example will make this clear.

Strength of standard 15.0 gm. of hemoglobin per 100 cc. of blood.

Comparison of cyanhemoglobin in colorimeter; Standard 10, Unknown 12.

Unknown has $\frac{12}{10}$ of 15.0 or 12.5 gm. of total blood pigment per 100 cc.

Gasometric determination of hemoglobin 10.0 gm. per 100 cc.

Therefore, sample has 12.5—10.0 or 2.5 gm. of methemoglobin per 100 cc.

DISCUSSION.

The deep orange-red color of the cyanhemoglobin is adapted to accurate color comparisons. Fiftyfold dilution of normal blood, containing approximately 15 gm. of hemoglobin per 100 cc., gives about the optimum depth of color. A 1:100 dilution gives too light a color and requires that the standard be set at 20.

TABLE II.

Hemoglobin + methemoglobin ob- served per 100 cc. (Colorimetric.)	Hemoglobin calculated per 100 cc. (Gasometric).	Methemoglobin observed per 100 cc.	Methemoglobin calculated per 100 cc.
gm.	gm.	gm.	gm.
6.89	6.74	0.15	0.00
6.89	6.43	0.31	0.46
6.86	6.15	0.69	0.71
6.62	4.56	2.17	2.06
6.58	0.20	6.54	6.38

The author has attempted to determine methemoglobin colorimetrically in blood by converting it, with the hemoglobin, into carbon monoxide hemoglobin, for estimation by the Palmer³ method, and into acid hematin for estimation by the Sahli principle; but both attempts were unsuccessful, since methemoglobin does not form products with carbon monoxide or acid which can be colorimetrically compared with the products formed from hemoglobin.

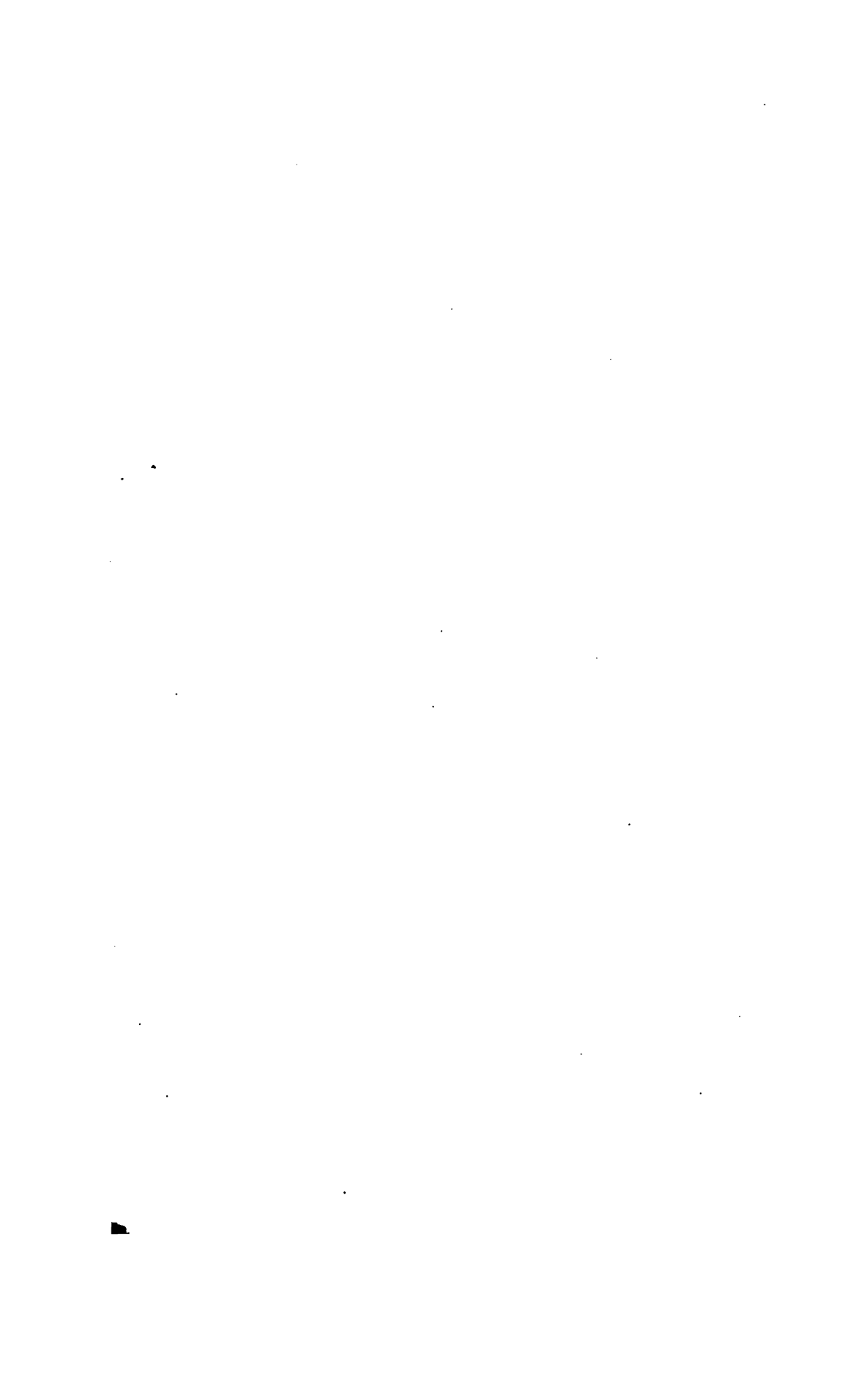
Results.—A solution of hemoglobin obtained from sheep's cells previously washed with saline was used. A gasometric determination of this solution showed it to contain 6.74 gm. of hemoglobin per 100 cc. Part of this solution was shaken for $\frac{1}{2}$ hour with an amount of potassium ferricyanide calculated to change all of it

³ Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

to methemoglobin. The hemoglobin content of this solution (gasometric) was 0.20 gm. per 100 cc. These two solutions were mixed in various proportions so as to make solutions containing varying but known amounts of hemoglobin and methemoglobin. The methemoglobin content was then determined as outlined above, using a standard from ox blood which had been prepared a few days before. The results are shown in Table II.

CONCLUSIONS.

A colorimetric method for the determination of blood pigments is given which is simple and rapid, and which, combined with a simultaneous determination of the hemoglobin by the gasometric method of Van Slyke, gives the methemoglobin content of the blood.



A STUDY OF THE NEPHELOMETRIC VALUES OF CHOLESTEROL AND THE HIGHER FATTY ACIDS. II.

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(Received for publication, December 8, 1919.)

Among the many influencing factors which govern the nephelometric value (N. V.) of fatty acids and cholesterol, I wish to discuss, in the present paper, certain important ones in their relation to saponification, because practically all quantitative fat determinations necessitate such a procedure. These factors consist of, on the one hand, substances which are essential to the determination, such as the reagents, or those which facilitate the production of colloidal suspensions, designated as protective colloids; and, on the other hand, conditions like heat, concentration, and the sequence of mixing the reagents in the development of turbidity.

However, before we apply either a procedure like saponification, or employ a protective colloid, we must ascertain its influence on the nephelometric values of the substances in question; an increase in the value is always to be desired, but a decrease is liable to render the determination less delicate, and therefore unsatisfactory as a micro method. Studies of this type are of interest not only as a preliminary step to the development of a new method, but also as a means of discovering the defects or proving the accuracy of a method already in use.

In the first paper,¹ where the suggested term nephelometric value was defined, the importance of acid concentration upon the nephelometric values of the fatty ingredients was emphasized and it was shown that fatty acids, as well as cholesterol, have specific nephelometric values. To overcome the difficulties caused by the fact that there exists a difference between the

¹ Csonka, F. A., *J. Biol. Chem.*, 1918, xxxiv, 577.

nephelometric values of the constituents of fat extracts, the first thought was to use a mixture for the standard, composed similarly to that of the unknown. The reliability of the result depends upon the extent of our success in reproducing the unknown mixture in the standard. Another suggestion would be to separate the ingredients of the fat extracts, although that would complicate the otherwise simple and rapid method. The ideal modification would be to employ such a technique as would render equal the nephelometric values of the various ingredients; the possibilities of regulating the nephelometric values by the factors referred to above will be demonstrated in the experimental part of this paper.

The present work is merely a preliminary study of the possibilities and as yet does not suggest any one procedure as preferable. The decision as to which of the above suggestions should be applied as the more useful in the nephelometric determination of fat will be considered in a later publication.

Methods.

Oleic acid and cholesterol were selected for the subjects of the present investigations, because they show the largest differences in their nephelometric values. The technique of these experiments, which is similar to that described in a previous article,¹ is such that the conclusions are directly applicable to the technique of Bloor's blood fat determination.

By the method described in the first paper,¹ the turbidity was produced by adding the HCl after the water had been added to the alcoholic solution of the fat; this was Technique A. In Technique B the sequence was reversed, the alcoholic solution of the fat being added to the water, and then the turbidity produced by adding the HCl as previously. In Series 1, oleic acid, cholesterol, and olive oil were used unmodified. In Series 2, the above mentioned substances were put through the saponification procedure by adding 2 cc. of *N* NaOH to the alcoholic solution in a 150 cc. beaker and placing this in a boiling water bath for 15 minutes to insure evaporation to dryness. To the residue were added 4 cc. of alcohol and the whole was warmed carefully to dissolve the fatty material. In those experiments, where the influence of gelatin upon the nephelometric value of oleic acid was studied, the amount of distilled water was substituted by a solution containing 50 mg. of gelatin. The final volume of the solution in which turbidity is produced is 50 cc. with 0.2 *N* acidity (HCl) and containing 4 cc. of 95 per cent alcohol. For comparison a permanent standard was employed, as previously described. Experiment A 1 with oleic acid was repeated many times during this work, as it is very im-

TABLE I.

Solution tested.	Amount.	Technique.	Turbidity produced.											
			5 min.		10 min.		15 min.		20 min.		25 min.		30 min.	
			Read- ing.	N. V.	Read- ing.	N. V.	Read- ing.	N. V.	Read- ing.	N. V.	Read- ing.	N. V.	Read- ing.	N. V.
	mg.		mm.		mm.		mm.		mm.		mm.		mm.	
Oleic acid.	2	A1	51.9	1.00	58.6	0.88	62.4	0.83	66.3	0.78	69.6	0.74	73.9	0.70
	2	A2	56.4	0.92	62.0	0.84	67.3	0.77	70.2	0.74	73.4	0.71	75.5	0.69
	2	B2*	50.2	1.03	56.5	0.92	60.4	0.86	63.0	0.82	64.0	0.81	66.5	0.78
Oleic acid and 50 mg. of gelatin.	2	A2	60.2	0.86	58.4	0.89	58.4	0.89	58.4	0.89	58.6	0.89	59.6	0.87
	4	B1	58.6	0.44	55.0	0.47	53.1	0.49	50.4	0.51	49.6	0.52	48.3	0.54
Cholesterol.	1	A1	49.6	2.09	48.0	2.16	47.9	2.17	47.3	2.19	47.0	2.21	47.6	2.18
	1	A2	51.1	2.03	49.4	2.10	48.2	2.15	48.1	2.16	48.7	2.13	49.2	2.11
	1	B1	48.3	2.15	47.1	2.20	46.4	2.23	46.3	2.23	46.4	2.23	46.4	2.23
	1	B2	44.3	2.34	44.3	2.34	44.1	2.35	44.1	2.35	44.1	2.35	44.9	2.31
Cholesterol and 50 mg. of gelatin.	2	B1	72.6	0.71	65.7	0.79	61.4	0.84	58.6	0.88	57.8	0.89	56.3	0.92

* B1 omitted as it is identical with A1.

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portant to be sure that the intensity of the nephelometric field (permanent standard) remains unchanged throughout. Readings were taken every 5 minutes for $\frac{1}{2}$ hour. The intensity of the nephelometric field used (permanent standard) is equal to the intensity of the turbidity produced by 2 mg. of the oleic acid after a lapse of 5 minutes, with the vernier set at the height of 51.9 mm. (Technique A, Series 1). The nephelometric values were calculated on that basis in all the experiments reported in this paper.

DISCUSSION.

As a result of the investigation described in the previous paper,¹ we conclude that in quantitative determinations it is erroneous to compare materials having different nephelometric values. From the experiments shown in Table I, it is evident that the saponification procedure alters the nephelometric value of the substance, and therefore it is advisable to treat the standard in the same manner as the unknown. In his first publication, Bloor² advocated the use of unsaponified triolein as a standard, later substituting for this oleic acid. By so doing, he overlooked the first source of error and disregarded the second.

The nephelometric value depends upon the conditions under which turbidity is produced; on account of the inconsistent and contradictory behavior of the different substances, the technique which is the more desirable must be previously determined in each individual case. For instance, we find that the nephelometric values of oleic acid and cholesterol in Series 1 are practically the same, whichever technique is employed, while Technique B gives higher values for Series 2. On the other hand, if we compare the result on olive oil³ which was selected to represent the glycerides in general (Table II), we find very little difference in Series 2, while Series 1 shows a pronounced difference; the values being much higher according to Technique A than to B.

By observing the changes of the nephelometric values within certain time intervals, it is interesting to note the difference between the changes shown by oleic acid and cholesterol; namely, the nephelometric value of the former is highest at the first reading and then decreases, while that of the latter shows a con-

¹ Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377; 1915, xxiii, 317.

³ The olive oil was dissolved in an alcohol-ether mixture for the stock solution.

TABLE II.

Solution tested.	Amount.	Technique.	Turbidity produced.											
			5 min.		10 min.		15 min.		20 min.		25 min.		30 min.	
			Read- ing.	N. V.	Read- ing.	N. V.	Read- ing.	N. V.	Read- ing.	N. V.	Read- ing.	N. V.	Read- ing.	N. V.
Olive oil.....	2	A1	51.3	1.01	56.9	0.91	60.2	0.86	62.3	0.83	65.9	0.79	68.2	0.76
".....	2	A2	57.5	0.90	63.9	0.81	69.0	0.75	72.3	0.72	76.6	0.68	78.7	0.66
".....	2	B1	61.8	0.84	68.5	0.76	75.0	0.69	80.4	0.65	85.6	0.61	88.6	0.60
".....	2	B2	57.8	0.90	65.3	0.79	70.4	0.74	73.2	0.71	76.6	0.68	81.4	0.64

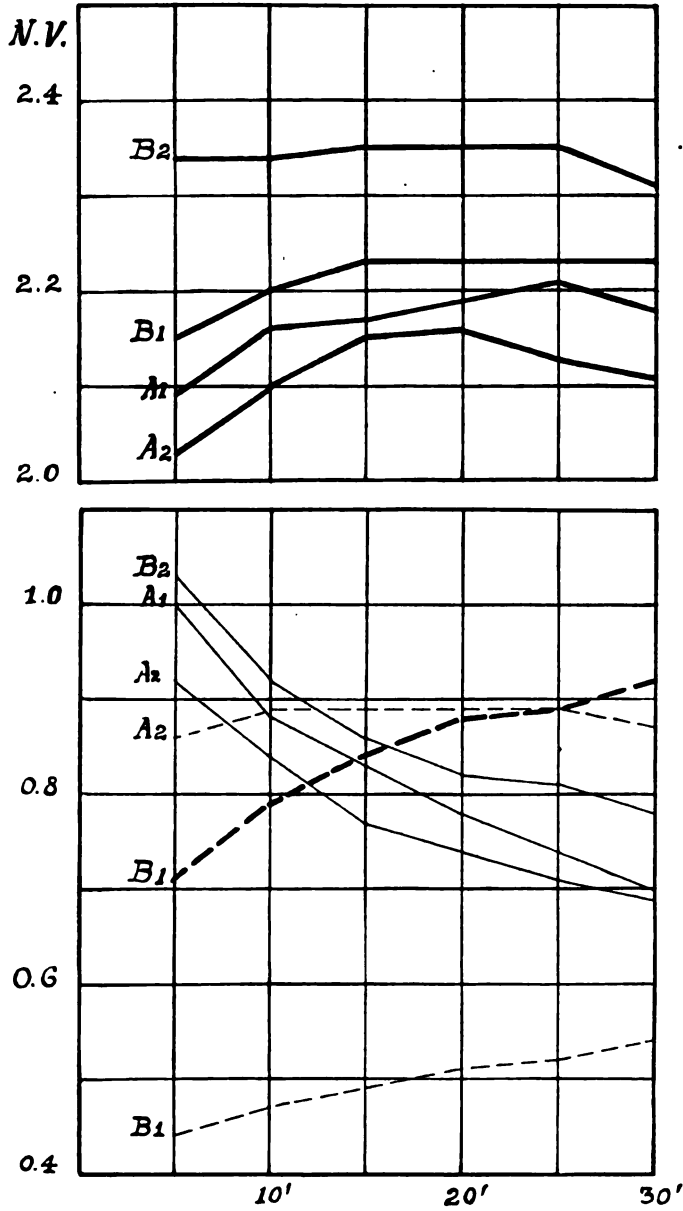


CHART 1. Changes of the nephelometric values (N. V.) within the first half hour.

Cholesterol	—	Oleic acid	—
" with gelatin	- - -	" " with gelatin	- - -

tinuous increase throughout the 30 minute interval. Chart I presents this variation graphically.

That it is possible to change not only the height, but also the entire direction of the curve is exemplified by the use of gelatin as a protective colloid, as first suggested by Murlin. With oleic acid (Series 1) the nephelometric value is more than 50 per cent lower after a lapse of 5 minutes and while this steadily increases it does not attain its maximum height within the 30 minute interval. In Series 2 the nephelometric values range between narrow limits, and are but slightly lowered. Determinations A1 and B2 were not successful; fairly constant readings could be obtained only in case of A2 and B1. Furthermore, applying Technique A the author was unable to produce a fine suspension of cholesterol in the presence of gelatin, even when the cholesterol was mixed with oleic acid, as the former separated into clumps on the surface. The presence of gelatin decreases the nephelometric value of cholesterol over 60 per cent when the turbidity is produced according to Technique B. A bluish tint is always observed in the nephelometric field whenever gelatin is used, which makes the reading somewhat difficult.

SUMMARY.

Oleic acid and cholesterol were the subjects of investigation. The nephelometric values of these substances were found to be influenced by the saponification procedure as well as by the addition of certain substances which alone, under similar circumstances, do not produce any turbidity. These influencing agents are exemplified by the use of gelatin as a protective colloid.

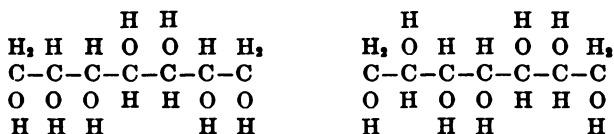
THE HEPTOSES FROM GULOSE AND SOME OF THEIR DERIVATIVES.

By F. B. LA FORGE.

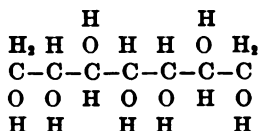
(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

(Received for publication, December 31, 1919.)

Of the sixteen theoretically possible heptoses of the *d* series, the following, as well as some of their derivatives, have been prepared and studied; α -glucoheptose,¹ β -glucoheptose,¹ α -mannoheptose,² β -mannoheptose,² α -galaheptose,³ and β -galaheptose.³ The number of possible heptitols, if optical isomerism is disregarded, obtained by reduction of these sugars is only ten, since the same heptitol may be formed from two different sugars. Thus, Peirce⁴ showed the identity of α -*d*-mannoheptitol with α -*l*-galaheptitol.



Likewise β -galaheptitol should be identical with one of the heptitols from gulose.



In the present paper the preparation from gulose of two new heptoses and their derivatives will be described and the identity of one of these with β -*d*-galaheptitol established.

¹ Fischer, E., *Ann. Chem.*, 1892, cclxx, 64.

² Fischer, E., and Passmore, F., *Ber. chem. Ges.*, 1890, xxiii, 2226.

³ Fischer, E., *Ann. Chem.*, 1895, cclxxxviii, 139.

⁴ Peirce, G., *J. Biol. Chem.*, 1915, xxiii, 327.

EXPERIMENTAL.

 α - and β -d-Guloheptonic Acids from Gulose.

80 gm. of gulose from the carefully purified hydrazone⁵ were dissolved in 200 cc. of water and slightly more than the calculated amount of hydrocyanic acid was added together with a few drops of ammonia to catalyze the reaction. The solution soon began to warm up, showing that reaction was taking place. After about 8 hours 25 gm. of sulfuric acid, diluted with a small amount of water, were added and the reaction products allowed to stand over night.

The solution was then strongly diluted, 150 gm. of barium hydroxide were added to it, and the ammonia was expelled by boiling. The barium was precipitated by a very slight excess of sulfuric acid and the barium sulfate removed by filtration with suction. The resulting solution contained the mixture of α - and β -guloheptonic acids. These could be obtained as a colorless syrup by decolorizing and evaporating the solution.

Barium Salt of α -Guloheptonic Acid.

For the preparation of this derivative the solution obtained from the above mentioned amount of gulose in 200 cc. of water was exactly neutralized while warm with a concentrated solution of barium hydroxide and then allowed to stand in the ice box. After several days the crystallization of the barium salt was judged to be complete. The yield was about 100 gm. The mother liquor contained the barium salt of the β -acid which will be referred to later. It was recrystallized from about twenty parts of hot water, from which it separates in large colorless plates generally grouped in rosettes.

⁵ The crude gulose obtained by reduction of gulonic lactone with sodium amalgam (Fischer, E., and Piloty, O., *Ber. chem. Ges.*, 1891, xxiv, 521) was dissolved in about eight parts of 65 to 70 per cent alcohol and to this solution was added slightly more than the calculated amount of phenylhydrazine base. Crystallization of the hydrazone began at once and was complete after several hours. The voluminous crystalline mass, which is difficult to filter, was subjected to strong pressure between several layers of filter paper and the resulting press-cake recrystallized from alcohol.

0.3550 gm. of substance gave 0.1402 gm. of BaSO₄.

	Calculated for C ₁₄ H ₂₂ O ₁₀ Ba. per cent	Found. per cent
Ba	23.37	23.22

Its optical activity could not be measured owing to its low solubility but seemed to be very slight.

α-d-Guloheptonic Acid.

The barium salt was dissolved in a convenient amount of hot water, the barium removed quantitatively with sulfuric acid, and the barium sulfate removed by filtration with suction. Upon concentration of the resulting solution the acid was obtained as a colorless syrup which did not crystallize. For reduction to the sugar the syrup was heated for a short time on the steam bath in order to convert as much as possible into the lactone.

Phenylhydrazide of α-Guloheptonic Acid.

This derivative was obtained very easily by heating a concentrated solution of α-guloheptonic acid, with about the theoretical amount of phenylhydrazine, on the steam bath for 1 hour. Upon cooling the reaction product crystallizes out and may be purified by recrystallization from 75 per cent alcohol. It forms long white needles which melt at 191–192°, uncorrected.

2.0656 gm. of substance in 25 cc. of H₂O rotated in a 2 dm. tube with D-light – 5.0° [α]_D²⁰ = – 15.38°.

α-d-Guloheptose.

30 gm. of the mixture of acid and lactone were dissolved in eight to ten parts of cold water and reduced with sodium amalgam.⁶ The reduction required about 200 gm. of 2.5 per cent amalgam. The solution was maintained acid by addition of dilute sulfuric acid from time to time.

The sodium salts were removed by pouring the hot concentrated solution into hot 95 per cent alcohol. The alcoholic solution was concentrated to a syrup, which was again taken up in a large volume of 95 per cent alcohol in order to separate the sugar as far as possible from the sodium salts.

⁶ La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, 1917, xxx, 68.

The alcoholic solution was then concentrated *in vacuo* to a small volume.

Crystallization of the sugar took place during evaporation. The yield was very good (9.0 gm.). It may be recrystallized from two parts of hot water or from ten parts of 60 per cent alcohol and forms rosettes of long needles. It is thus much less soluble than most sugars and is only slightly sweet. The sugar melts at 185–187°, uncorrected.

0.7616 gm. of substance in 25 cc. of H₂O rotated in a 2 dm. tube at 20° and D-light after about 8 minutes – 19.5° to the left, after 35 minutes – 14.0°, and became constant at – 11.5°. $[\alpha]_D^{20} = -65.65^\circ$.

0.1703 gm. of substance gave 0.1017 gm. of H₂O and 0.2521 gm. of CO₂.

	Calculated for C ₇ H ₁₂ O ₇ per cent	Found. per cent
C.....	40.00	40.36
H:.....	6.66	6.57

α-Guloheptitol.

5 gm. of *α*-guloheptose were reduced with sodium amalgam in the usual manner. The solution was kept slightly alkaline most of the time. When the copper reducing power had practically disappeared, which required 3 days action of the reagent, the reaction product was poured into hot alcohol to remove the sodium sulfate and the solution concentrated to a syrup. This crystallized, on rubbing with absolute alcohol, to a semisolid, waxy mass. After washing with alcohol the product was dissolved in three or four parts of water and hot absolute alcohol added in amount sufficient to produce permanent turbidity.

Under these conditions the compound crystallized in rosettes of hard prisms, which melted at 138–141°, uncorrected. After a second recrystallization it showed no change in melting point.⁷

⁷ Peirce⁴ has prepared *β*-galaheptitol and gives the melting point at 141–144° after the substance had begun to soften at 138°. He states that the melt did not become clear until the temperature was raised to 190°. From the method used by him for the preparation of *β*-galaheptitol, it seems extremely likely that his product may have been contaminated with some of the epimeric *α*-galaheptitol. There seems to be no reason to suppose that *β*-galaheptitol and *α*-guloheptitol are not identical.

0.1009 gm. of substance gave 0.1480 gm. of CO_2 and 0.0682 gm. of H_2O .

	Calculated for $\text{C}_7\text{H}_{14}\text{O}_7$ per cent	Found. per cent
C	39.60	39.96
H	7.64	7.51

1.3280 gm. of substance in 25 cc. of H_2O showed no rotation in a 2 dm. tube which could be detected.

1.0624 gm. in 25 cc. of saturated aqueous borax solution rotated in a 4 dm. tube with D-light $+ 0.68^\circ$ to the right.

β -Guloheptonic Acid and β -Guloheptose.

The filtrate from α -guloheptonic acid barium salt, above referred to, was further concentrated and cooled to cause the separation of as much of the α -compound as possible. The filtrate was diluted, the barium removed quantitatively with sulfuric acid, and the solution of the β -acid, together with some of the α -compound, concentrated to a syrup. This syrup was heated on the steam bath to convert as much as possible of the acid into lactone and then reduced under the conditions already described.

After separation of the sodium salts by means of twice repeated treatment with hot 98 per cent alcohol the sugar was obtained upon evaporation of the solvent as a syrup. This syrup still contained some of the α -compound which could be removed to a large extent by dissolving the mixture in a small amount of glacial acetic acid and allowing the almost insoluble α -sugar to crystallize out.

The syrup resulting after removal of the acetic acid was not entirely pure but was used in this condition for reduction.

Reduction of β -Guloheptose.

The syrupy β -guloheptose was reduced according to the usual method in slightly alkaline solution. The reduction was continued until the copper reducing power of the solution had almost completely disappeared, after which the salts were removed and the reaction product obtained as a syrup. Since this syrup showed no tendency to crystallize, it was transformed into the benzal derivative.

Benzal-β-Guloheptitol.

This derivative was obtained with ease by agitating equal parts of 70 per cent sulfuric acid, benzaldehyde, and the syrupy mixture containing the heptitol. It was washed with water and alcohol and dried at 100°. It melted at about 260° with decomposition.

β-Guloheptitol.

One part benzal-β-guloheptitol was boiled for about 1½ hours with about twelve parts 60 per cent acetic acid. The solution was diluted and excess of the reagent, together with most of the benzaldehyde, was removed by repeated extractions with ether. It was then concentrated to a small volume and again extracted with the same solvent. Upon standing the syrup resulting from evaporation of the final solution deposited crystals of the heptitol which were washed with glacial acetic acid and recrystallized from 95 per cent alcohol. The compound melted at 129°, uncorrected. It was again recrystallized and melted at 128–129°, uncorrected.

0.1025 gm. of substance gave 0.1495 gm. of CO₂ and 0.0653 gm. of H₂O.

	Calculated for C ₇ H ₁₄ O ₇ . per cent	Found. per cent
C	39.60	39.98
H	7.64	7.09

0.3175 gm. of substance in 5 cc. of saturated aqueous borax solution showed no appreciable rotation in a 1 dm. tube.

STUDIES ON THE SECRETION OF GASTRIC JUICE.

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(Received for publication, December 2, 1919.)

In a previous article (1), on the secretion of gastric juice in fever, it was reported that the concentration of chlorides in gastric juice was constant or slightly reduced. In this present paper I wish to emphasize the relation between the secretion of total chlorides in gastric juice and the secretion of free hydrochloric acid. Most of the data for this paper were obtained at that time but until now have remained unpublished.

The gastric juice was obtained, as previously, from the stomach of dogs with a Pavlov pouch. The animals were kept on a standard diet. Their temperature varied as the result of the production of fever by intravenous injection of solutions of sodium nucleinate or *Bacillus prodigiosus*. The free hydrochloric acid was determined by titrating 1 cc. of the gastric juice with $N/40$ NaOH, dimethylaminoazobenzene being used as indicator. The total chlorides were determined by the method of McLean and Van Slyke (2).

The results given were obtained from 175 chloride determinations of gastric juice on seventeen different animals. The free hydrochloric acidity varied from 0.0 to 0.48 per cent while the total chlorides varied from 0.39 to 0.54 per cent. In examining Charts 1, 2, 3, and 4, it will be noted that the curves for the secretion of free hydrochloric acid do not correspond to the curves for the secretion of total chlorides, although the percentage of total chlorides is always greater than the percentage of free acid, as should be expected. The curve for the total chlorides is almost a straight line with just slight deviations, while the curve for the free acid varies markedly. In some places, however, both curves run nearly parallel, while at other times there is practically no relation between them.

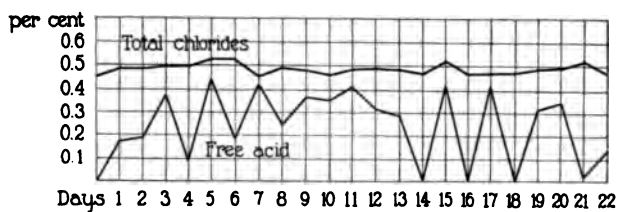


CHART 1. Showing the relation between the secretion of free HCl and the secretion of total chlorides on Dog 2.

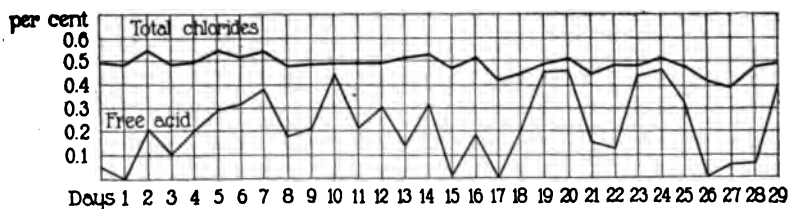


CHART 2. Showing the relation between the secretion of free HCl and the secretion of total chlorides on Dog 5.

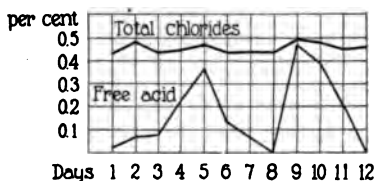


CHART 3. Showing the relation between the secretion of free HCl and the secretion of total chlorides on Dog 20.

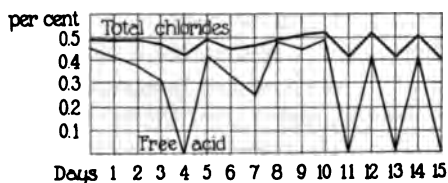


CHART 4. Showing the relation between the secretion of free HCl and the secretion of total chlorides on Dog 22.

From these results it appears that the chlorides of gastric juice are secreted at a more or less constant rate and in all likelihood independently of the hydrochloric acid secretion. In other words you may get just as high a percentage of total chlorides in a sample of gastric juice when the free acidity is 0.0 as when it is 0.35 per cent. This may indicate that the hydrochloric acid is not secreted as such but is probably formed by the secretion of some substance, containing the chlorine ion, which is transformed in the lumen of the stomach or by stomach mucosa to the free acid. This, however, is not a new idea (3). If free hydrochloric acid were secreted as such by the gastric glands, one would expect that with a decrease in the free acidity or a total absence of the free acidity there would be associated with it a marked fall in the total chlorides, but this does not seem to be true. The chlorides are secreted more or less constantly regardless of the free acidity of the gastric juice.

On the other hand, these results may coincide with those of Pavlov (4), and Rehfuess and Hawk (5). Pavlov states that the gastric juice as it flows from the glands possesses a constant acidity; variations are due to secondary neutralization. The apparent constant rate of secretion of chlorides may mean that the gastric juice is secreted with a constant acidity and that the variations in the end-product are due to neutralization by mucus or the alkalinity of the stomach mucosa. This point might be proved by studying a case of achylia and determining the chloride content in the juice; such a case has not been available.

Another factor in the secretion of HCl is the relation between volume and acidity. Pavlov states that, when secretion of the gastric gland is abundant, then the acidity is higher than when the secretion is scant. This is due to the fact that when the juice flows rapidly the acidity is higher, because there is less change of the mucus or alkaline mucosa to neutralize it. This fact is very well illustrated by Charts 5, 6, and 7 in which the volume of gastric juice (5 hours secretion) is charted with the free acidity. It will be noted that free acidity is almost directly proportional to the total volume. This agrees with Pavlov's statement that a large volume of gastric juice, which is secreted rather rapidly, has a higher acidity than a smaller volume, which is secreted more slowly.

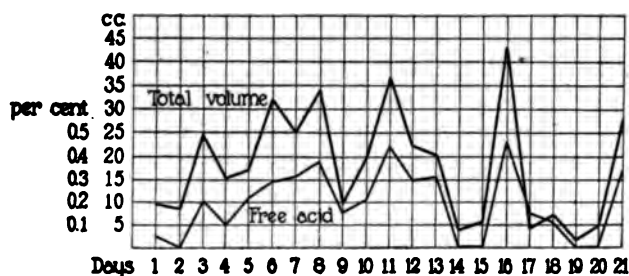


CHART 5. Showing the relation between the secretion of free HCl and the total volume on Dog 2.

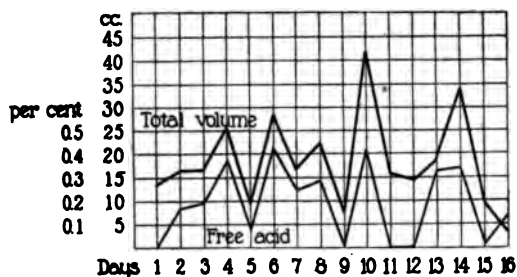


Chart 6. Showing the relation between the secretion of free HCl and the total volume on Dog 5.

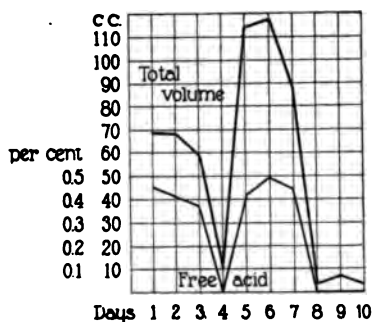


CHART 7. Showing the relation between the secretion of free HCl and the total volume on Dog 22.

SUMMARY.

1. The total chlorides of gastric juice from the dog are secreted more or less constantly regardless of the free acidity of the gastric juice. They vary from 0.39 to 0.54 per cent.

2. This may corroborate Pavlov's view that gastric juice is secreted with a constant acidity and that variations are due to secondary neutralization.

3. A large volume of juice secreted rapidly has a higher acidity than a smaller volume secreted slowly, other factors remaining constant (confirmatory of Pavlov).

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DIRECT QUANTITATIVE DETERMINATION OF POTASSIUM AND SODIUM IN SMALL QUANTITIES OF BLOOD.

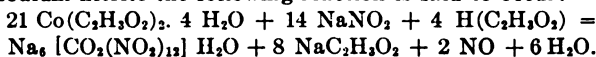
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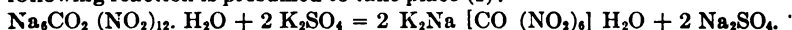
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INTRODUCTION.

When a solution of a cobalt salt in dilute acetic acid is added to a solution of sodium nitrite the following reaction is said to occur:



The solution becomes dark brown and oxides of nitrogen are evolved. When these are removed by aeration and a little of the resulting reagent is added to a fairly concentrated solution of any potassium salt containing a little acetic acid, an orange-yellow precipitate is formed at once. The following reaction is presumed to take place (1):



These phenomena were first reported by de Koninck (2) and a few months later by Curtman (3). They observed independently that the reagent does not react with Ca, Mg, Na, Ba, Sr, Zn, Fe, sulfate, nitrate, or chloride ions. It is therefore possible to detect potassium in the presence of these ions. The ammonium ion gives a precipitate with the reagent and phosphates, unless present in very small amounts, will do likewise. Addie and Wood (4) isolated and analyzed the potassium sodium cobalti-nitrite compound formed under definite experimental conditions. They showed that the precipitate has a constant composition represented by the formula $\text{K}_2\text{Na} [\text{Co} (\text{NO}_2)_6] \cdot \text{H}_2\text{O}$, and determined its solubility to be rather a little less than 1 part in 20,000. If the concentration of the potassium salt is not less than 0.5 per cent, the precipitate is formed at once, settles rapidly, and the particles are large enough to be retained by a Gooch filter.

Where it is desired to determine large amounts of potassium as the cobalti-nitrite compound, the method of Addie and Wood (4), that of Drushel (5), or some modification of either may be used (1, 6, 7). The extreme insolubility of the precipitate in water, its sensitiveness under appropriate conditions for minute amounts of potassium, and the indifference of the reagent to other ions usually present in physiological fluids make it suitable for the determination of such small amounts of potassium as are found in blood.

The first attempt to determine potassium in small amounts of blood as the potassium sodium cobalti-nitrite compound was made by Hamburger (8). By his method precipitation is accomplished in a centrifuge tube, the lower end of which is drawn out to a capillary and graduated. The volume of the precipitate is measured in this tube, each graduation being equal to 0.0001 gm. of potassium. The precipitation requires at least 16 hours for completion. No analyses of serum, plasma, or blood are given.

Greenwald (7) has described a composite method for the determination of potassium, sodium, calcium, and phosphates in 90 gm. of blood. The potassium is precipitated as the cobalti-nitrite compound. More recently Clausen (9) reported a method for the determination of potassium in small quantities of blood. The figures reported justify all that the author claims for his method. The precipitation method is essentially that advocated by Drushel. In some preliminary experiments which we performed in 1917 this procedure was tried. In agreement with Bowser (10) it was found that in attempting to concentrate the fluid after adding the reagent decomposition of the precipitate at times took place, resulting in the formation of what appeared to be cobalt acetate. Hence we discarded it for the simpler procedure described below.

The methods described in this paper were devised for the study of changes in the concentration of sodium and potassium in the blood of children. The results of these studies, carried on for more than 2 years, will be published later. In the meanwhile it has been thought desirable to put the methods themselves on record.

Potassium Method.

1 cc. of blood, 3 to 5 cc. of clear plasma, or an equal amount of serum is dried in a platinum dish over the steam bath, then in the incubator at 110°C. for about $\frac{1}{2}$ hour. The dish or crucible is then placed in a flat-bottomed quartz dish, 10 cm. in diameter and 6 cm. deep, in the bottom of which are placed several pieces of porcelain. The outer dish is then heated with the low flame of a large Meker burner until fumes begin to come off. The heating is continued until no more fumes are given off, when the flame is turned on full until the charred material is immobile. The large dish is then covered with a quartz plate and heating continued until the material is completely ashed.¹ The platinum dish is

¹ The ashing often proceeds rapidly at first, then some residual carbon is left which does not readily become oxidized. At this stage the ash is dissolved in a little concentrated hydrochloric acid, evaporated over the steam bath, then dried in the incubator at 105°C., and heated until a white crystalline ash is obtained.

then removed, allowed to cool, and the ash dissolved in 0.5 cc. of water with the aid of one or two drops of glacial acetic acid. 0.5 to 1 cc. of sodium cobalti-nitrite reagent is then added, drop by drop, with stirring and the mixture allowed to stand for at least 10 minutes. During this time a Gooch crucible is prepared. One or two pieces of hardened filter paper are placed at the bottom, then asbestos emulsion is poured in, and the pad sucked dry. When finished the pad should be at least 2 mm. in thickness and should be washed with a large quantity of water. The precipitate is transferred to the wet pad. The suction should be regulated so that the water runs through drop by drop; the rest of the precipitate is washed onto the pad with small portions of cold water. It is then washed repeatedly with small portions of water until the washings return perfectly clear. Precipitate and pad are then transferred *en masse* to a 50 cc. beaker, and the crucible is washed with a little water.² The paper is then removed with a forceps and washed clean with distilled water. Not more than 10 cc. of water need be used for complete transference of asbestos pad and precipitate. 25 cc. (an excess) of 0.01 N potassium permanganate and 5 cc. of 25 per cent sulfuric acid are added. The mixture is stirred, heated over the steam bath for just 3 minutes, and sufficient 0.01 N oxalic acid is then added to decolorize the solution completely. The material is at once titrated back to a permanent pink with 0.01 N KMnO_4 solution. The total number of cc. of 0.01 N KMnO_4 — number of cc. of 0.01 N oxalic acid \times 0.071 = mg. of potassium in the sample.

A blank may be done but the correction is insignificant as a rule.

Details of the Procedure.

Collecting Sample.—Blood is collected by puncture of the median basilic, median cephalic, or external jugular vein. Whole blood is collected directly into a weighed platinum crucible and rapidly weighed. Plasma or serum may be collected under oil as in the Van Slyke and Cullen determination of the CO_2 -combining power of blood plasma (11). To obtain plasma, potassium-free am-

² The pad need not be sucked dry. By means of a glass rod it may be dislodged by a half turn and can then be transferred as a whole including the entire precipitate.

monium oxalate or oxalic acid may be used as anticoagulant. Although ammonium salts react with sodium cobalti-nitrite, the ammonia is completely volatilized during the ashing. To obtain serum, the blood is collected under oil in a centrifuge tube and allowed to remain in the ice box until the serum has separated. If the blood is collected in this manner or in a clean, dry test-tube the danger of hemolysis will be reduced to a minimum.³

Ashing.—The method of ashing which has already been described was first used by Stolte (12). It is simple and fairly rapid. There is no loss of salts through volatilization. Chemically pure potassium or sodium chloride may be thus heated for 48 hours without loss. 1 mg. of potassium similarly treated may be recovered quantitatively. It is important to do such control tests to be sure that the experimental conditions have been duplicated.

Asbestos.—Dilute permanganate solutions when heated on the water bath for some time undergo change. This process is hastened by the presence of asbestos and is not due to organic matter that may be present in the asbestos. (This is all oxidized in a preliminary blank titration.) Nevertheless, if the oxidation is not continued beyond 3 minutes as described, the presence of the asbestos has no influence on the titration. We have found the use of barium sulfate suspension superfluous and its presence makes transference of the precipitate more difficult. Asbestos emulsion may be made by prolonged digestion of a good asbestos with strong nitric and hydrochloric acid for about 8 hours. The asbestos is separated from the supernatant fluid by filtering through a Buchner funnel. It is then washed with water until washings are no longer acid to litmus, then suspended in water, digested with 10 per cent NaOH for an equal period of time, filtered off, washed with dilute hydrochloric acid and then with water, sucked dry, suspended in water, and shaken until a uniform suspension is obtained.

³ Owing to the large amount of potassium present in corpuscles even a moderate amount of hemolysis introduces considerable error.

Preparation of the Reagents.

25 per cent sulfuric acid is made by diluting chemically pure concentrated sulfuric acid with water. It should be tested with permanganate for the presence of organic matter.

The Sodium Cobalti-Nitrite Reagent.—This may be prepared according to the method of Addie and Wood (4). A somewhat easier method is the following, first suggested by Hamburger (8). This yields a less concentrated reagent.

Solution A.—50 gm. of cobalt nitrate crystals (J. T. Baker) are dissolved in 100 cc. of water and to this solution 25 cc. of glacial acetic acid are added.

Solution B.—50 gm. of c.p. sodium nitrite (potassium-free) (J. T. Baker) are dissolved in 100 cc. of water. Mix six volumes of Solution A and ten volumes of Solution B. An evolution of nitric oxide gas occurs at once. Air is drawn through the solution until all the gas has passed off. The reagent is then allowed to stand in the ice box for at least 24 hours. It is best filtered before using.

The cobalt nitrate and sodium nitrite need be weighed only roughly. If kept in the ice box the reagent will keep for at least a month and often much longer. Although 1.5 cc. of reagent will precipitate as much as 20 mg. of potassium (Hamburger), we have found it best to use about 0.5 cc. where 1 mg. of potassium or less is thought to be present. For quantities between 1 to 3 mg., 1 cc. of reagent will suffice.

0.01 N Potassium Permanganate Solution.—0.01 N potassium permanganate solution was prepared from a N or 0.1 N solution by appropriate dilution. The solution was standardized against a known 0.01 N oxalic acid solution. The latter was made from a N oxalic acid solution.

Na, Ca, Mg, Fe, sulfates, chlorides, and nitrate do not interfere. Ammonia must be removed. Although such small amounts of phosphates as occur in normal blood do not interfere with the accuracy of the potassium determination, nevertheless, as has recently been shown (13), the inorganic phosphates may be markedly increased in the blood serum in certain pathological conditions. Hence it may be necessary to remove them (see Table II). The following method has been found satisfactory. The solution

of blood or serum ash acidified with hydrochloric acid is heated on the steam bath for a few minutes. 2 cc. of 2 per cent BaCl_2 solution are added drop by drop followed after a few minutes by 0.5 cc. of concentrated ammonia. 15 cc. of a saturated solution of ammonium carbonate in a mixture of equal volumes of concentrated ammonia and 95 per cent alcohol (14) are added drop by drop with stirring. After $\frac{1}{2}$ hour the precipitate, which contains practically all the barium, calcium, magnesium, sulfate, and phosphate, is filtered through ash-free filter paper and washed several times with the precipitating reagent.

When the filtrate which contains the sodium and potassium has been evaporated to a small volume, a few drops of concentrated hydrochloric acid are added. Evaporation to dryness is continued and completed in the incubator at 110°C . The residue is then heated to constant weight by the method of Stolte and the sodium and potassium are weighed as the chlorides. When combined with the cobalti-nitrite method for potassium, this procedure may be used as a rapid method for the indirect determination of sodium.⁴

Protocols.

Three solutions were made up.

Solution 1 contained 1 mg. of potassium per cc. as chemically pure potassium chloride.

Solution 2 had the following composition:

	gm.
NaCl	6.4389
KCl	1.4184
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	2.4992
MgSO_4	0.3695
CaCl_2	0.1836
H_2O up to 1,000.0 cc.	

Solution 3 contained

	gm.
NaCl	1.287
KCl	0.56
Ca as CaCl_2	0.10
Mg as MgSO_4	0.20
H_2O up to 200.0 cc.	

All chemicals were "Kahlbaum zur Analyse" or prepared by recrystallization of "Baker's Analyzed." $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ was recrystallized and air-dried (17).

⁴ It is also possible to weigh the combined chlorides of sodium and potassium, determine the total chlorine, and calculate the amount of potassium and sodium (15, 16).

TABLE I.
Analysis of Four Samples of Solution 1.

Sample No.	K present.	K found.
	mg.	mg.
1	1.00	0.99
2	1.00	0.98
3	1.00	1.00
4	1.00	1.01

TABLE II.
Analysis of Six Samples of Solution 2.

Sample No.	K present.	K found.*
	mg.	mg.
1	0.74	0.84
2	0.74	0.84
3	2.22	2.45
4	2.22	2.45
5	1.00	1.13
6	1.00	1.07

* The high results are no doubt due to the presence of phosphate. Solution 3 contains same ingredients but no phosphates.

TABLE III.
Analysis of Three Samples of Solution 3.

Sample No.	K present.	K found.
	mg.	mg.
1	1.00	0.98
2	1.00	1.05
3	1.00	1.06

TABLE IV.
Recovery of Potassium Added to Serum.

	K present.	K found.
	mg.	mg.
K in serum.....	0.94	
K added as KCl.....	1.48	
Total.....	2.42	2.39
K in serum.....	0.94	
K added as KCl.....	1.48	
Total.....	2.42	2.42

*Sodium Method.**Introduction.*

The usual method for determining sodium in the presence of other ions is to isolate sodium and potassium as chlorides or sulfates, weigh the combined salts, then determine potassium as the chloroplatinate, the cobalti-nitrite, or the perchlorate, and calculate the amount of sodium from the data thus obtained. Such methods are indirect, very tedious, and obviously cannot be used to determine small quantities.

The general solubility of sodium compounds in the common solvents has tended to discourage attempts to devise a direct sodium method. Fenton (18) found that the sodium salt of dihydroxytartaric acid is insoluble in cold water and used this as the basis for a direct sodium method. Although recommended by Sutton,⁵ the method has not attained any degree of popularity probably because of the difficulty in preparing the reagent and the solubility of the precipitate.

In 1910 Ball (19) described a direct method for the determination of sodium based on the formation of an insoluble sodium cesium bismuthi-nitrite ($9 \text{ CsNO}_3, 6 \text{ NaNO}_2, 5 \text{ Bi(NO}_2)_3$). The reagent is expensive, does not keep well, and large quantities must be used for each determination. The precipitation must be accomplished in a special vessel in the absence of air and requires 48 hours for completion. No suitable washing solution has been found which does not dissolve some of the precipitate.

The use of potassium pyroantimonate for the detection of sodium has been known for a long time. When a solution of this reagent is added to a fairly concentrated solution of a sodium salt, a precipitate is formed, either at once or on standing, which is crystalline in nature and has the composition $\text{Na}_2\text{H}_2\text{Sb}_2\text{O}_7, 6 \text{ H}_2\text{O}$ (20). Precipitation is favored by neutral or slightly alkaline reaction, and is accelerated by the addition of alcohol. The precipitate is very slightly soluble in water. The presence of K, Mg, Ca, SO_4 , PO_4 , or chloride ions does not interfere with the reaction. *Ammonium salts also form an insoluble pyroantimonate and must therefore be removed.*

⁵ Sutton (15), p. 65.

Technique of the Sodium Method.

The ash of 1 or 2 cc. of blood, serum, or plasma obtained as described for the potassium method is dissolved in water, in a platinum dish, using 0.5 cc. of water for each cc. of serum, plasma, or blood. Solution may be aided by the addition of a drop or two of *N* hydrochloric acid. The solution is then made slightly alkaline with freshly prepared 10 per cent KOH solution. 15 cc. of the reagent and one-fifth of the entire volume of absolute alcohol⁶ are then added. Precipitation occurs at once. The mixture is stirred, allowed to stand for at least 2 hours (preferably over night), then transferred to the wet pad of a weighed Gooch crucible as in the potassium method. It is then washed four or five times with 3 cc. portions of 30 per cent alcohol, dried at 110°C., cooled in a desiccator, and weighed. 1 mg. of sodium yields 11.08 mg. of precipitate. All reagents should be tested for the presence of sodium and ammonium salts, especially the potassium hydroxide. A blank determination should be done and the result subtracted from the Na determination in the sample. Most of the laboratory reagents will give a slight precipitate with the potassium pyroantimonate reagent.

Preparation of the Reagents.

Potassium pyroantimonate (J. T. Baker, c.p. analyzed chemicals), 2 gm. of the powder, is added to 100 cc. of boiling water in a 350 cc. Pyrex flask and heating continued until no more dissolves. It is then cooled rapidly under the tap and 3 cc. of 10 per cent KOH are added, and the solution is stirred and filtered. The clear filtrate constitutes the reagent.⁵ Although I have been able to precipitate sodium with a reagent that had been kept in the ice box for 2 months, I have, nevertheless, always prepared the solution fresh each time. The potassium antimonate need be weighed only roughly and the rest of the preparation takes but a few minutes. 10 per cent KOH should preferably be free of both sodium and ammonium salts. Alcohol-washed KOH contains relatively little of these. The exact content, if any is

⁶ The addition of too much alcohol will precipitate some of the reagent which is itself not very soluble and hence the results will be too high.

TABLE V.
Analysis of a Solution of C. P. Sodium Chloride.

Sodium as sodium chloride.	
Present.	Found.
mg.	mg.
5.91	5.87
5.91	6.02

TABLE VI.
*Analysis of Samples of Solution 2.**

Sodium.	
Present.	Found.
mg.	mg.
6.34	6.53
6.34	6.48
6.34	6.40
6.34	6.43

	gm.
*NaCl.....	6.4389
KCl.....	1.4184
Na ₂ HPO ₄ ·2H ₂ O.....	2.4992
MgSO ₄	0.3695
CaCl ₂	0.1836
H ₂ O up to 1,000.0 cc.	

TABLE VII.
Recovery of Sodium Added to Serum.

	Present.	Found.
	mg.	mg.
Sodium in serum.....	14.90	
“ added as sodium chloride.....	6.34	
Total.....	21.24	22.32
Sodium in serum.....	14.90	
“ added as sodium chloride.....	6.34	
Total.....	21.34	21.16
Sodium in serum.....	19.40	
“ added.....	6.34	
Total.....	25.74	25.30

present, should, of course, be determined and a correction made for the amount used. This solution should also be made fresh or kept so that it will neither absorb ammonia nor dissolve sodium.

CONCLUSIONS.

Simple methods for the direct quantitative estimation of sodium and potassium in small quantities of blood, serum, or plasma have been described that are usually accurate to within 3 per cent of the theory and often closer (see Tables I, III, V, and VI).

By using the indirect sodium method described above, both sodium and potassium may be determined on 3 cc. of serum with an error not exceeding ± 5 per cent.

Results obtained by both methods for sodium are in satisfactory agreement.

The potassium content of normal human serum varies between 16 and 22 mg. per 100 cc. of serum. The sodium content has been found in both normal children and adults to vary between 280 and 310 mg. per 100 cc. of serum.

Sodium or potassium added to serum may be recovered almost quantitatively (see Tables IV and VII).

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NUTRITIVE VALUE OF THE PROTEINS OF THE BARLEY, OAT, RYE, AND WHEAT KERNELS.*

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Investigators are at present in accord in the conviction that none of the common cereal grains alone or even a mixture of them suffices to afford satisfactory nutrition. The reason for this is to be found primarily in a deficiency in certain of the essential dietary components, notably specific inorganic elements and fat-soluble vitamins. The inadequacy with respect to such needed factors may be complete or partial; in either event nutrition, especially during growth, will be impaired. The particular deficiencies just referred to can readily be offset by supplying otherwise the items needed. The foremost remaining dietary factor in these seeds is the protein. In some of them the proportion of protein calories—the nutritive ratio—is rather low. Furthermore, it has been demonstrated conclusively that some of the individual proteins, like zein (maize), gliadin (wheat), and hordein (barley), for example, are chemically defective and, correspondingly, physiologically inadequate proteins. Hence a misconception of the possible value of the cereals as sources of protein has developed in the minds of some persons owing to their failure to realize that in the form in which these grains are most commonly fed the sum total of their various proteins must be taken into account.

*The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

In attempting a further study of the relative nutritive value of the cereal proteins we have already expressed the fundamental view-point as follows:

"The comparison of the cereals, which resemble each other closely in being composed in major part of starch and other carbohydrates and are somewhat alike in respect to the presence or absence of different inorganic nutrients and vitamins, rests in a large measure upon the relative equivalence of their unlike proteins for the uses of nutrition. An ideal comparison, from this standpoint, requires a diet adequate and equivalent in respect to all other essentials, both organic and inorganic, known to constitute a perfect food when suitable protein is supplied therewith."¹

For the white rat which we have employed in these investigations it is essential to have a considerable concentration of protein in the diet if suitable growth is to be attained. This is particularly true when the quality of the protein is somewhat inferior so that relatively large quantities are needed to furnish the indispensable minimum of the limiting amino-acids. Furthermore, if any considerable proportion of other foods is added to furnish the lacking indispensable non-protein factors, the protein content of the resulting dietary mixture becomes correspondingly diluted.

With these facts in mind we attempted to prepare protein concentrates from various cereal grains by removing as much as possible of the starch of the seeds. The preliminary results obtained with such concentrates, supposedly representing the bulk of the total protein in the respective cereals, were not entirely satisfactory.¹ There was an unlike inclusion of indigestible residues—presumably carbohydrates—in the different products; so that these inevitable conditions rendered accurate comparisons on a protein basis somewhat inconclusive.

The concentrates used represented only about 80 per cent of the total proteins of the seed. At that time we thought that in order to get the accurate food intakes which alone permit the drawing of definite conclusions regarding the relative nutritive values of the different proteins, it was necessary to use a food mixture rich in fat. Inasmuch as the cereals are low in protein, the addition of sufficient fat to make the food of the proper consistency increased its calorific value so greatly that too little

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 521.

protein was eaten to promote satisfactory growth. In our investigation of the nutritive value of wheat² we found that it was possible to obtain accurate food intakes on diets containing as little as 5 per cent of fat. This was done by adding to the mixture of finely ground wheat, salts, and butter fat enough water to make a dough which the rats would not scatter. This food contained only about 3.75 calories per gm. as compared with our ordinary fat-rich foods which contain on the average about 5 calories per gm. Consequently the animals ate much more liberal quantities of the 5 per cent butter fat food than they did of the fat-rich food, so that although the *percentage* of protein in the former food might be relatively low, the *total* consumption of protein on this food was as great as that on the fat-rich, protein-rich foods ordinarily used. This method, therefore, made it possible for us to study the unchanged total proteins of the various seeds at different protein levels and compare their nutritive value according to the numerical method which we have previously described.³

We have resumed the investigations by feeding the entire cereal grains, finely ground, along with an adequate salt mixture and sufficient butter fat to supply the fat-soluble vitamine. We have assumed, on the basis of evidence obtained by both ourselves and others, that the quantities of the entire cereal grains used by us would supply sufficient water-soluble vitamine.

The method employed in this series of comparative experiments was to feed the finely ground seed mixed with 3 per cent of a suitable salt mixture, 5 per cent of butter fat, and, where necessary, enough corn-starch to make the total protein of the ration 5, 8, or 10 per cent, approximately. In this way the proteins of barley, oats, rye, and wheat were compared.

Each day a little more than enough of the finely ground food for one day's feeding was mixed with a quantity of water sufficient to make a soft dough, and the mixture was packed into the food cups. The next morning the food that remained was dried in an oven. At the end of the week the residues were collected, weighed, and their weight was deducted from that of the dry

² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

³ Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxvii, 223.

ingredients fed. Care was taken to make the daily excess of food so small that inaccuracies involved in drying the residues were reduced to a minimum.

Barley.

In preparing the foods intended for the study of the nutritive value of the barley proteins special care was taken to grind the grain, including the husks or glumes, to a very fine powder. The precise composition of the various food mixtures used was as follows:

	10 per cent protein food.	8 per cent protein food.	5 per cent protein food.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Ground barley.....	92	72.0-75.5	45-47
Salt mixture*.....	3	3.0	3
Butter fat.....	5	5.0	5
Starch.....	0	20.0-16.5	47-45

*The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The results of the feeding experiments with these mixtures, as exhibited in the gains of body weight of growing rats, are shown in Charts I, II, and III.

These experiments leave no doubt of the adequacy of the barley *proteins* as a whole in the nutrition of growth; for several of the rats grew to large adult size without any other source of protein than that derived from this cereal. Some of the animals on the higher percentages of barley protein even surpassed the normal rate of growth on ordinary mixed food. With the lower concentration of barley in the food the deficit of protein appears as a factor limiting the rate of growth, though even on the lowest percentage not inconsiderable gains were made. The possibility of growth upon a diet in which barley supplied the protein was earlier indicated in our experiments with barley concentrates.¹ These tests are less conclusive than the present series because the rations included, during part of the period, a small amount of brewer's yeast containing protein which possibly supplemented the barley protein so as to increase the rate of growth.

In a valuable contribution on the dietary qualities of barley, Steenbock, Kent, and Gross⁴ have reached the conclusion that

"The protein content of barley (13.6 per cent) is too low for continued growth at the normal rate. When reduced in amount to 8.1 per cent, the small amount of growth that results is soon followed by a decline. 5.4 per cent just about suffices for maintenance.

The primary growth determinant in barley is inorganic salts. Of secondary importance, but no less urgent, are protein and fat-soluble vitamins."

Steenbock, Kent, and Gross⁴ have reproduced the growth curve of one rat (No. 25, Chart 10) which made excellent gains on barley, unsupplemented by other protein. Why their other rats on the same diet did not grow so well is not clear. Our animals ate the rations in liberal amounts, as the food intakes which we have measured indicate.

The composite growth curve published by McCollum, Simmonds, and Parsons⁵ for rats on a diet in which all the protein, amounting to 9 per cent of the ration, was derived from barley also fails to indicate a degree of gain comparable with that attained by our animals on rations containing 8 per cent of barley proteins. It should be noted, however, that our food mixture was not like that of McCollum, Simmonds, and Parsons which contained "seed = to 9 per cent protein; NaCl, 1.0; CaCO₃, 1.5; butter fat, 3.0; dextrin to 100."

We have not made observations on the total length of life of animals which have grown up upon a diet furnishing proteins from a single seed like barley, or upon their ability to produce and rear young. We believe that there are too many other nutritive factors involved in successful nutrition to enable us, upon the basis of our present knowledge, to charge any failures of nutrition in the second generation to chemical inadequacy of protein solely, although it is quite conceivable that the level of protein metabolism represented by a relatively high or low content of protein in the ration over a long span of life may have a pronounced effect upon the maternal functions. It seems to us,

⁴ Steenbock, H., Kent, H. E., and Gross, E. G., *J. Biol. Chem.*, 1918, **xxxv**, 61.

⁵ McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, **xxxvii**, 155, see Chart I, p. 164.

however, that if an animal is able to attain adult size upon a diet which furnishes protein from a single source, the nutritive value of this protein is clearly established. Our experiments with barley have demonstrated the possibility of attaining large size where proteins from this source alone were available—provided the total intake of food and consequently of protein and energy was adequate. In view of such positive results, in contrast with the less successful growth obtained in experiments made by others and conducted perhaps under less favorable conditions, it seems hazardous to venture a strict comparison of the nutritional value of proteins from the records of different investigators without more precise information of a quantitative nature regarding the relations of body gain to protein intake where the energy and other essential factors are strictly comparable.

In explanation of the less satisfactory growth observed on the diets containing the lower concentration of barley proteins it might be assumed that a deficiency of water-soluble vitamine, due to the smaller content of the seed in the ration, was the underlying cause. Judging from the evidence secured by Steenbock, Kent, and Gross⁴ regarding the content of water-soluble vitamine in barley, it is not probable that a lack of the latter was the cause of poorer growth in our experiments with diets containing only 5 per cent of barley protein. The rats on the 5 per cent barley protein diets ate as much total food, *i.e.* calories, as those of the same size on the higher concentrations of protein. The quantities probably were as large as the animals could consume. Hence we conclude that the deficiency of protein was the direct outcome of its low content in the ration. We have, however, directed new experiments to the solution of this problem by furnishing a ration low in barley protein but unquestionably adequate in water-soluble vitamine. The diet was the same as in the earlier series, with the addition of approximately 18.6 mg. daily, apart from the food, of a protein-free vitamine preparation from yeast.⁶ This quantity, which supplemented the water-soluble vitamine in the barley already present in the 5 per cent protein food, had been shown in test experiments to be adequate for promoting food intake and growth on our standard food

⁴ Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

mixtures. As will be apparent from Chart XIII, recording these experiments, no nutritive advantage accrued from this vitamin

TABLE I.
Barley.

Protein in food.	Rat.	Initial weight.	4 week period.			10 week period.		
			Gain in weight.	Total food intake.	Gain per gm. pro- tein eaten.	Gain in weight.	Total food intake.	Gain per gm. pro- tein eaten.
<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
10	5717 ♂	71	69	404	1.7	129	973	1.3
10	5716 ♂	64	64	376	1.7			
10	5701 ♂	72	63	334	1.9	120	950	1.3
10	5251 ♂	66	50	320	1.6	74	764	1.0
10	5709 ♂	70	49	340	1.4	117	983*	1.2
10	5256 ♂	70	43	352	1.2	92	829	1.1
10	5267 ♂	68	37	263	1.4	78	744	1.0
8	5406 ♂	58	78	397	2.5	187	1,178	2.0
8	5407 ♂	74	58	391*	1.9	122	1,090*	1.4
8	5654 ♂	85	57	364	2.0			
8	5649 ♂	71	55	408	1.7			
8	5659 ♂	66	46	297	1.9	114	917	1.6
8	5410 ♂	58	42	328	1.6	86	745*	1.4
8	5662 ♂	76	40	312	1.6	112	964	1.5
5	5427 ♂	74	43	423	2.0	84	1,043	1.6
5	5437 ♂	75	35	337*	2.0	49	807*	1.2
5	5660 ♂	75	33	406	1.6	79	1,091	1.4
5	5432 ♂	69	31	347	1.8			
5	5707 ♂	70	26	311	1.7	64	861	1.5
5	5656 ♂	75	23	316	1.5	49	871*	1.1
5	5661 ♂	67	19	241	1.6	47	645	1.5
5	5423 ♂	66	17	228	1.5	51	630	1.6

Barley + Vitamine.

5	6211 ♂	70	11	234	0.9			
5	6248 ♂	70	20	328	1.2			

*The records for the food intakes in these experiments were unsatisfactory.

supplement. The food intakes (Rats 6211 and 6248) showed no noteworthy increase; nor was the gain per gm. of protein eaten more favorable (see Table I), hence we must still ascribe the slow

growth on the 5 per cent barley protein ration to the comparatively small quantity of protein afforded by the diet.

Pearled Barley.—We have undertaken a few feeding experiments with pearled barley. This preparation differed from the entire barley kernel in lacking the husks or glumes and the greater part of the outer coats of the seed; *i.e.*, the bran. The food mixture consisted of

	<i>per cent</i>
Pearled barley.....	92
Salt mixture*.....	3
Butter fat.....	5

* The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

These ingredients were mixed with a little water and baked in an oven.

TABLE II.
Pearled Barley.

Protein in food.	Rat.	Initial weight.	1 week period.		
			Gain in weight.	Total food intake.	Gain per gm. protein eaten.
<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
8	5005♂	83	34	302	1.4
8	4961♂	76	24	270	1.1
8	5010♂	67	13	237	0.7

The outcome of the experiment is shown in Chart IV and Table II. The growth obtained was relatively slight in comparison with the more adequate gains on foods of a similar caloric nature containing 8 per cent of protein from the *entire* barley grain. The addition of vitamine in the form of yeast did not improve the results. It is not unlikely that the milling process has removed fractions of the barley protein, residing in the outer layers of the grain, which supplement the less effective proteins of the barley endosperm. In a similar way it is known that the total proteins of wheat are superior to the proteins of the endosperm of that cereal.

Oats.

In the present series of experiments to ascertain the nutritive value of the oat proteins finely ground, commercial oat groats⁷ were employed in preparing the foods. The various mixtures had the following composition.

	10 per cent protein food.	8 per cent protein food.	5 per cent protein food.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Oat kernels.....	70	57	35.5
Salt mixture*.....	3	3	3.0
Butter fat.....	5	5	5.0
Starch.....	22	35	56.5

* The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The results of the feeding experiments with these mixtures, as exhibited in the gains of body weight of growing rats, are shown in Charts V, VI, and VII.

The successful growth of several of the animals to large size must, we believe, be interpreted to indicate that the total protein of the oat kernel can furnish all the essential nitrogenous units if the intake of food and its concentration of protein are adequate. Very low concentrations of protein in a diet necessitate such large intakes of food to provide enough protein for growth at a normal rate that the energy intake would be inordinately large. Rats will not consume such excesses of food calories; hence, when they satisfy their energy requirement by ingestion of a food relatively poor in protein, growth is slowed in proportion to the quantity (and quality) of the *protein* actually consumed. This is evidently the limiting factor in the experiments with rations containing only 5 per cent of oat proteins.

As a rule our rats for some reason did not eat the oat foods so readily as the barley rations. The food intakes recorded in Tables I and III indicate this. Herein may lie the explanation of the failure of a considerable number to grow well. Some of

⁷ The oat groats were the entire kernels of the oat seed from which the husks or glumes were removed. This material was kindly supplied to us by Mr. Cutting of the Quaker Oats Company.

TABLE III.

Oat.

Protein in food.	Rat.	Initial weight.	4 week period.			10 week period.		
			Gain in weight.	Total food intake.	Gain per gm. pro- tein eaten.	Gain in weight.	Total food intake.	Gain per gm. pro- tein eaten.
<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
10	5700♂	65	38	248	1.5	80	616	1.3
10	5688♂	66	26	215	1.2	83	590	1.4
10	5677♂	71	21	202	1.0			
10	5693♂	70	21	209	1.0			
8	5428♂	70	48	306	2.0	115	824	1.7
8	5433♂	58	45	313*	1.8	77	702*	1.4
8	5442♂	66	30	246	1.5			
8	5461♂	66	22	204	1.3	40	501	1.0
8	5501♂	65	21	204	1.3			
8	5486♂	58	20	189	1.3	27	438	0.8
8	5464♂	69	19	235	1.0	48	638*	0.9
8	5500♂	61	19	184	1.3	24	447	0.7
8	5424♂	63	17	211	1.0	31	484	0.8
5	5484♂	67	36	338	2.1	74	839	1.8
5	5443♂	66	29	262	2.2	54	656	1.7
5	5444♂	71	27	249	2.2	59	616	1.9
5	5485♂	62	24	261	1.8	68	746	1.8
5	5490♂	63	22	250	1.8	36	608	1.2
5	5438♂	65	21	252	1.7	38	589	1.3
5	5511♂	66	21	246	1.7			
5	5429♂	80	16	294	1.1	60	746	1.6
Oat + Vitamine.								
5	6206♂	69	15	238	1.3			
5	6213♂	70	27	226	2.4			
5	6234♂	69	25	279	1.8			

* The records for the food intakes in these experiments were unsatisfactory.

our experiments (Rats 5442, 5433, 5461, 5424, 5486, 5500, 5438, and 5490) were terminated without waiting to observe a possible subsequent improvement, because we were primarily concerned at the time with the comparison of the protein efficiency of different cereals under conditions of comparable energy intake.

Our long continued and successful results make the hypothesis of a toxic factor improbable.

It was possible that the content of water-soluble vitamine might be the limiting factor in those cases where the food intake was not large. Experiments by McCollum and his collaborators⁸ indicate that 60 per cent of rolled oats in a mixture similar in energy value to our ration can supply enough of this vitamine to permit good growth. We have, nevertheless, repeated the experiments during a period of 4 weeks by furnishing a ration low in oat protein but unquestionably adequate in water-soluble vitamine.

These trials were comparable to those already described with barley (page 280), the same supply of protein-free yeast vitamine being employed. Chart XIII, as well as the statistical data (Table III), fails to indicate any advantage in the larger intake of water-soluble vitamine, so that the results with the 5 per cent oat protein food must be ascribed to the comparatively small content of protein in the diet.

McCollum⁹ and his associates have recorded numerous observations upon the dietary value of the oat kernel. He states:

"We have not yet been able to supplement oats with purified food ingredients and attain optimum results, when the oat kernel constituted from 70 to 80 per cent of the food mixture. Gelatin combined with oat proteins forms a much better protein mixture than do casein and oat proteins. . . . We have not yet determined the cause, but it is evident that a high intake of oats over a long period causes injury to the rat. This is true also for the cow, and I believe also for swine."¹⁰

Our experience with respect to the value of casein and gelatin respectively has been recorded elsewhere.¹

Why we have obtained very considerable growth upon oat diets containing 5 per cent of protein whereas McCollum and

⁸ McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916-17, xxviii, 492; 1917, xxix, 341.

⁹ McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916-17, xxviii, 483; 1917, xxix, 341. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 347. McCollum, E. V., *The newer knowledge of nutrition*, New York, 1919, 163.

¹⁰ McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379.

Simmonds¹¹ rarely secured maintenance upon foods of essentially similar energy content containing either 4.5 or 6.75 per cent of oat proteins (see their Chart 7) is not clear. The only conspicuous difference in the rations fed lies in the use of starch in our diets instead of dextrin and agar in the food mixtures used by McCollum and Simmonds. Their experiments in which large supplements of casein or other proteins were used have less significance in estimating the comparative value of the different cereal proteins; the quantities of the supplementary protein employed have presumably in themselves been sufficient to promote growth independently of the cereal protein used. The confusion now existing in the literature of nutrition regarding the rôle of the oat proteins *per se* (aside from any possible detrimental factor in the kernel as a whole) may be attributed to the conflicting statements published. Thus we read in one place, "The oat kernel seems to contain proteins of a poorer quality than either the maize or wheat kernel."¹² Again we read,

"The protein of the oat kernel has a slightly higher value for growth than has that of either wheat or corn, but the amount furnished by 90 per cent of rolled oats is below the optimum for the support of growth in a rapidly growing species."¹³

Or again we are told that "the oat proteins are distinctly better than those of wheat, maize, or rice."¹¹

In a recent report from the Agricultural Experiment Station of the University of Wisconsin, referring to the effect of organic nutrients on animal growth and reproduction, it is stated:

"In every case where the oat plant was fed miserably poor offspring has resulted; for instance, with oatmeal and oat straw and butterfat; oatmeal, casein, butterfat and oat straw. This would seem to indicate that the trouble is not due to the poor protein or low vitamine content, but is a matter of actual deficiency in the mineral elements in the ration. Where the ration has been fortified with either calcium acetate or wood ashes, normal offspring has resulted. Further experiments are necessary to con-

¹¹ McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 362.

¹² McCollum, E. V., Simmonds N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 342.

¹³ McCollum, E. V., *The newer knowledge of nutrition*, New York, 1919, 163.

firm this, but it is probably true that the main mineral constituent which is lacking is calcium. . . . Oats fed with corn stover and other roughages carrying a high calcium content gave no trouble whatever."¹⁴

We shall, therefore, refer later to the indications offered by our own strictly comparable tests of the comparative growth-promoting value of different cereals.

Rye.

For the experiments to ascertain the nutritive value of the rye proteins the finely ground entire kernels were employed in food mixtures of the following composition.

	10 per cent protein food.	8 per cent protein food.	5 per cent protein food.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Whole rye.....	80.0-85.4	64.0-69.5	40.0-42.7
Salt mixture*.....	3.0	3.0	3.0
Butter fat.....	5.0	5.0	5.0
Starch.....	12.0-6.6	22.5-28.0	49.3-52.0

* The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol.Chem.*, 1919, xxxvii, 572.

The results of the feeding experiments, exhibited in gains of body weight of growing rats on these mixtures, are shown in Charts VIII, IX, and X, and Table IV.

Out of all these trials with male rats, only three, Nos. 5447, 5446, 5465, have reached a body weight of 200 gm. Many of the rats, notably those on the foods containing the higher percentages of rye, have grown well for a considerable period, during which their food intakes, as recorded later in this paper, were reasonably large and, as will be subsequently shown, the efficiency of the proteins for growth was comparable with that of the other cereal proteins included in the present study. Had our investigations been confined to a few weeks or months we probably should have failed to realize the exceptionally large mortality which subsequently involved our rye-fed animals. It is unlikely that these untoward results are due to some intercurrent disease prevalent

¹⁴ Russell, H. L., and Morrison, F. B., *Univ. Wisconsin Agric. Exp. Station, Bull.* 302, 1919, 55.

TABLE IV.
Rye.

Protein in food.	Rat.	Initial weight.	4 week period.			10 week period.		
			Gain in weight.	Total food intake.	Gain per gm. pro- tein eaten.	Gain in weight.	Total food intake.	Gain per gm. pro- tein eaten.
<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
10	5733 ♂	67	48	308	1.6	105	739	1.4
10	5737 ♂	71	33	275	1.2	84	723	1.2
10	5732 ♂	70	30	235	1.3	84	651	1.3
10	5731 ♂	71	30	288	1.0	69	686	1.0
8	5447 ♂	82	44	328	1.7	102	884	1.4
8	5522 ♂	67	43	295	1.8	73	688	1.3
8	5524 ♂	74	42	291	1.8	76	724	1.3
8	5448 ♂	69	37	304	1.5	75	728	1.3
8	5487 ♂	82	36	320	1.4	60	699	1.1
8	5523 ♂	75	34	304	1.4	68	709	1.2
8	5449 ♂	76	27	277	1.2	81	711	1.4
8	5446 ♂	84	26	287	1.1	77	734	1.3
5	5553 ♂	71	34	344	2.0	57	882*	1.3
5	5552 ♂	74	20	339	1.2			
5	5462 ♂	75	19	320	1.2	55	763	1.4
5	5465 ♂	73	19	293	1.3	51	737	1.4
5	5557 ♂	66	19	288	1.3	44	830	1.1
5	5556 ♂	69	18	249	1.4	39	718	1.1
5	5471 ♂	71	17	245	1.4	42	600	1.4
5	5472 ♂	69	17	274	1.2	44	669	1.3
Rye + Vitamine.								
5	6223 ♂	70	21	283	1.5			
5	6226 ♂	69	17	278	1.2			
5	6224 ♂	70	15	274	1.1			

*The records for the food intakes in these experiments were unsatisfactory.

in our colony, because in that event a comparable number of deaths might have been expected among the other groups of cereal-fed animals simultaneously being investigated. The seemingly good nutrition of the rye-fed animals in the earlier periods of their life makes it unlikely that the proteins *per se* are chargeable with the untoward results subsequently exhibited by this

group of animals as a whole. What other deleterious factor, if any, may be present we are as yet unable to state.

It is not likely that the poor growth secured on the 5 per cent rye protein mixtures was due to a lack of water-soluble vitamine, inasmuch as the special series of experiments wherein 18.6 mg. of protein-free vitamine preparation from yeast were supplied daily yielded no better results (Chart XIII). The low protein content of the ration was evidently the limiting factor in growth.

The only comparable experiments which we have discovered in the literature are by McCollum, Simmonds, and Parsons⁵ who state:

" . . . such cereal grains as maize, rye, and barley contain proteins of such values that when fed at 9 per cent of the food mixture, supplemented with respect to certain salts and fat-soluble A, young rats are able to grow at approximately half the normal rate."

In the composite growth chart of male animals, presented by these authors, a body weight of 200 gm. was attained. In further experiments in which rye, to the extent of 50 per cent (equivalent to 6 per cent of protein from rye) of the ration, was supplemented by flaxseed oil meal or millet seed as a further source of protein, females grew fairly well and produced young.¹⁵ The diet was regarded as not quite satisfactory because of "the poor quality of its proteins, and shortage of fat-soluble A." Nevertheless these animals received in the diet 2 per cent of butter fat, a quantity which McCollum reports elsewhere¹⁶ "to be sufficient for the maintenance of good growth when all other dietary factors are of good quality." The only further reference to the rye proteins which we have noted is the statement of McCollum, Simmonds, and Parsons¹⁷ according to which

" . . . it appears that rye and flaxseed proteins in this proportion [rye proteins 6 per cent, flaxseed proteins 3 per cent] are nearly if not quite equal in value for growth to the proteins of milk."

¹⁵ McCollum, E. V., *The newer knowledge of nutrition*, New York, 1919, 175.

¹⁶ McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 162.

¹⁷ McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 160.

Wheat.

The wheat mixtures used in these experiments were prepared from the whole wheat kernel ground in the laboratory. They had the following composition.

	10 per cent protein food.	8 per cent protein food.	5 per cent protein food.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Whole wheat.....	92	70	51
Salt mixture*.....	3	3	3
Butter fat.....	5	5	5
Starch.....	0	22	41

* The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

That growth to large adult size can be completed at a normal rate on the food containing 92 per cent of wheat has already been demonstrated by us.¹⁸ Several litters of young were secured from females on this diet. The growth of rats kept on the wheat food of lower protein content is shown in Charts XI and XII. Several of the experiments with 8 per cent wheat protein were terminated after a few weeks of observation without reference to the final outcome. The growth of the remainder, though fairly vigorous at first, soon slowed.

The young rats of the second generation on the 10 per cent wheat protein food¹⁹ failed to grow with normal vigor. Thus of one litter from Rat 4681 ♀, three young, 5075♂, 5076♂, 5077♂, died at the ages of 6, 8, and 12 months respectively without having attained a maximum weight of more than 75, 75, and 62 gm. respectively. All the young of a second litter died at an early age. Out of a litter from Rat 4577 ♀, two died very young and 5325 ♀, 5326 ♀, 5327 ♀, 5328 ♀, 5330♂, and 5331♂ survived for some time, having reached a maximum body weight of 107, 45, 57, 32, 40, and 48 gm., at 270, 88, 117, 102, 151, and 137 days

¹⁸ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557, Chart II.

¹⁹ The curves of growth of Rats 4577 and 4681, which bore these young, have been published. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919 xxxvii, 557, Chart II.

TABLE V.
Wheat.

Protein in food.	Rat.	Initial weight.	4 week period.			10 week period.		
			Gain in weight.	Total food intake.	Gain per gm. pro- tein eaten.	Gain in weight.	Total food intake.	Gain per gm. pro- tein eaten.
<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
10	4669♂	60	45	322	1.4	114	810	1.4
10	4689♂	63	44	322	1.4	102	823	1.2
10	4680♂	68	39	287	1.4	92	784	1.2
8	5463♂	66	40	345	1.4	84	873	1.2
8	5469♂	65	35	346	1.3	77	873*	1.1
8	5381♂	62	34	332	1.3	83	848	1.2
8	5470♂	67	33	323	1.3	78	815	1.2
8	5382♂	67	28	274	1.3			
8	5457♂	62	23	234	1.2	46	596	1.0
8	5375♂	61	20	275	0.9	52	753	0.9
5	5711♂	76	18	289	1.2	41	714	1.1
5	5694♂	66	14	262	1.1	31	600	1.0
5	5676♂	71	9	254	0.7	29	606	1.0
5	5690♂	69	7	237	0.6	29	547	1.1
5	5416♂	61	4	207	0.4			
5	5415♂	65	4	211	0.4			
5	5399♂	76	4	271	0.3			
5	5398♂	71	4	242	0.3			
Wheat + Vitamine.								
5	6227♂	69	25	264	1.9			
5	6229♂	70	22	317	1.4			
5	6222♂	70	7	242	0.6			

* The records for the food intakes in these experiments were unsatisfactory.

respectively. Whether this outcome was due to a protein deficiency—which seems less likely—or to some other factor cannot be decided with certainty at present. McCollum, Simmonds, and Pitz²⁰ also state that they have not been able to make up a ration containing wheat proteins only which was adequate for

²⁰ McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916-17, xxviii, 211.

rearing of the young. They add: "Over a wide range of protein content growth approximated the normal, but pronounced injurious effects of the ration were revealed in the reproduction records only." Some of the young (see their Chart 9) appeared normal except for their diminutive size. We have had a similar experience.²¹ Incidentally we may note, however, that we have successfully raised a third generation of fertile rats on a diet in which the protein was derived solely from commercial wheat embryo.²²

Whether the slow growth of the rats on diets containing only 5 per cent of wheat protein was due to a lack of water-soluble vitamin in the 51 per cent of wheat contained in the food mixtures can be judged by comparison with the further results obtained by furnishing a ration equally low in wheat protein, but unquestionably adequate in water-soluble vitamin. This was derived from additions of 18.6 mg. per day of protein-free vitamin preparation from yeast and employed as in the experiments with 5 per cent barley proteins (page 280). If one may judge from the food intakes and increments of body weight (Table V and Chart XIII)—which were essentially of the same order as those in the other 5 per cent wheat protein food series—the limiting factor in the wheat protein trials is to be found in the low protein content of the ration.

The Comparative Nutrient Efficiency of the Entire Proteins of the Barley, Oat, Rye, and Wheat Kernels.

The growth of our rats, on diets essentially comparable except in respect to content and source of the cereal *proteins* contained therein, show the possibilities of nutrition when any one of four commonly used cereal grains furnished the protein. To formulate a tenable comparison of the relative nutritive value of these proteins in growth it is necessary, as we have frequently pointed out before, to know the food intake. The criteria for such experiments have already been discussed elsewhere by us.³

²¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 596.

²² The food mixtures and history of the parent animals are described in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557, Chart XIV.

The tabular summaries give the essential facts in the case of those animals for which the data are reasonably accurate. In many trials, particularly with the wheat mixtures, so many animals spilled food as to render the statistics for food intake incomplete; hence they have been rejected in every case. The figures recorded are the selected residual complete data from a large number of trials. The growth results have been calculated from animals beginning at a body weight of about 70 to 80 gm. Calculations have been made to include the subsequent first 4 weeks; and likewise the first 10 weeks, during which a rat normally more than doubles its body weight. The composition of the foods from the standpoint of calorie value was substantially the same, starch being substituted for cereal whenever the content of the latter was reduced in the ration; hence the energy intake in the experiments with foods containing different protein concentrations must have been practically proportional to the total intake of food. The graphic records of the body weights of all these animals will be found in Charts I to XIII.

If the figures representing gains per gm. of protein eaten are contrasted for the first periods of 4 weeks a slight advantage might seem to accrue to the barley proteins. This advantage is scarcely apparent, however, when the longer period (10 weeks) is taken into account. Obviously the number of trials is far too small to permit the use of such limited statistics for computation of averages. Until a far greater refinement of the method is secured it would appear, on the whole, that the proteins of the four cereals studied are not widely different in their efficiency in promoting growth. In contrasting the different groups of animals from a standpoint of their subsequent history, however, it seems as if the barley-fed animals have, if anything, grown best.

The comparative equality of the four types of cereal proteins just recorded becomes the more striking when the gains which they promote per gm. of protein consumed are contrasted with those secured within comparable periods of growth by the use of proteins from other sources. Thus, in feeding rats of similar initial weight for periods of 8 weeks, gains per gm. of protein eaten amounted to more than 2.3 gm. for lactalbumin and 1.7 gm. for casein. The advantage of the addition of more efficient supplementary protein to the cereal protein has been discussed elsewhere.²

In somewhat similar experiments on the comparative nutritive value, in growth, of proteins of wheat endosperm (flour and wheat gluten) with and without supplements of animal protein (meat, milk, egg), the best gains per gm. of protein eaten were not much larger, during a 4 week period, than those here reported for the four cereals. The outcome with the wheat endosperm proteins alone was decidedly poorer, as the following summary shows.

Summary.*

	Per cent of protein in food.	Gain of body weight per gm. of protein.
		gm.
Flour + egg.....	14.8	2.00
	10.3	1.80
“ + milk.....	14.8	1.67
	10.3	1.73
“ + meat.....	14.8	1.73
	10.3	1.47
“ + “ + yeast.....	10.3	1.66
“ + gluten.....	14.8	0.50

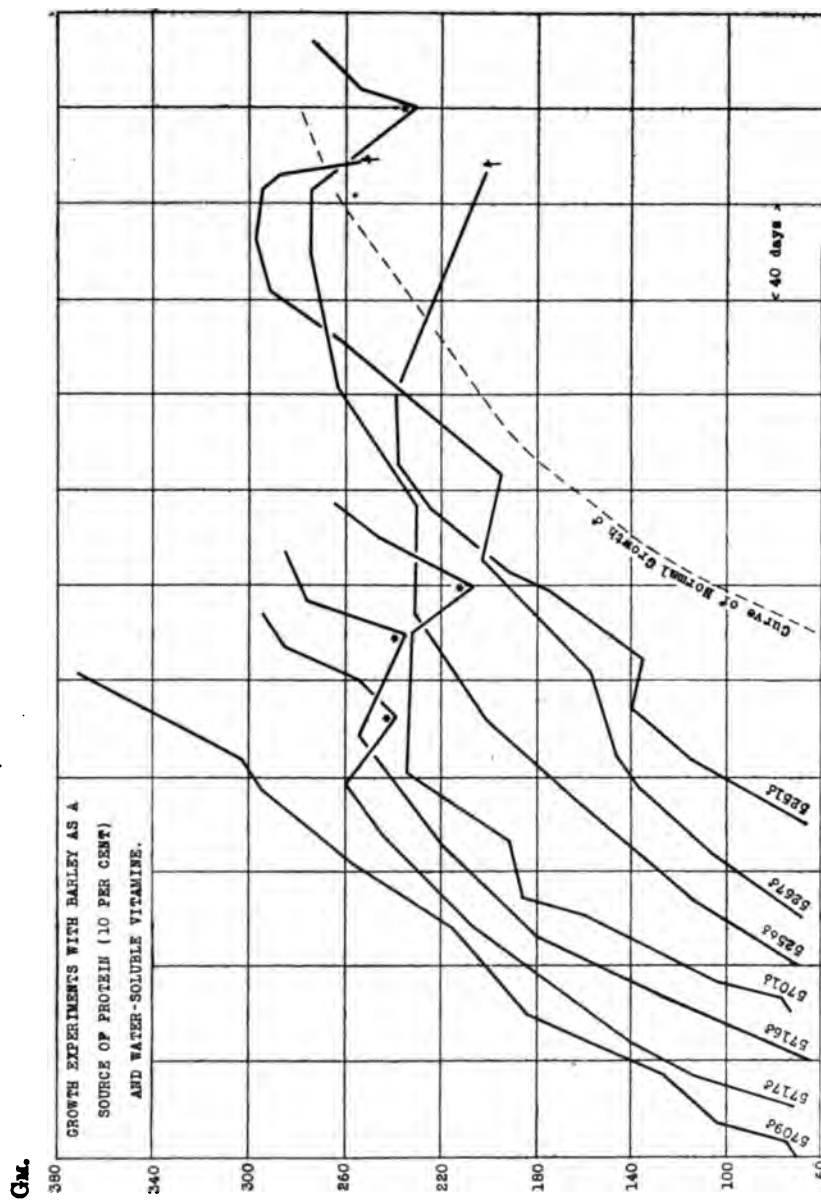
* Quoted from Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 584.

We were surprised to find that the *efficiency* of the *entire* wheat kernel, as well as that of the other cereal grains studied, without supplementary proteins, was so far superior to that ascertained by us earlier in the study of the *endosperm*. The utilization data secured by us testify to the unexpected availability for growth of the proteins of these whole cereals. This is in harmony with the recent findings of Sherman and his collaborators²³ in the study of the maintenance metabolism of adults as shown by nitrogen balance experiments on diets in which the proteins of oats and maize contributed a large portion of the protein intake. They assert:

²³ Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383. Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 301. Sherman, H. C., Winters, J. C., and Phillips, V., *J. Biol. Chem.*, 1919, xxxix, 53.

" the proteins of oats and maize are of virtually equal nutritive efficiency; and this is true whether the proteins in question constitute practically the sole nitrogenous food or are supplemented by a constant small amount of milk protein. That nitrogen equilibrium was maintained with such low protein intake in the latter experiments, and was so closely approached in the former, shows that the proteins of both maize and oatmeal were very efficiently utilized in the maintenance metabolism of these healthy adults. . . . For the purposes of practical dietetics equal weights of oat and maize proteins may be regarded as essentially equal in value, and even the minimum amount of milk which can possibly be regarded as permissible in the light of our present knowledge of nutrition, will apparently so supplement the proteins of either the maize or oat kernel as to make them function with an efficiency comparable with that of the average protein of mixed diet in the maintenance metabolism of man."

The ideal of equivalent calorie intakes during comparable periods of time in the growth experiments to compare the efficiency of proteins from different sources has by no means been realized in the trials which we have recorded in this paper. At best our experiments show the possibility of normal growth for long periods where no other proteins than those furnished from these cereals are available. They also indicate a surprisingly good efficiency in the most favorable experiments; but inasmuch as many ultimate failures of growth and well being, and numerous deaths, were encountered in the later periods of many of the experiments with some of these cereals it will be necessary to take into account such unknown factors as may have been responsible for these ultimately unfavorable results. We have observed sufficient, successful, prolonged growth in the case of all the cereal studies to make it unlikely, not to say improbable, that the protein factor is responsible for this untoward outcome of many of the cereal experiments.



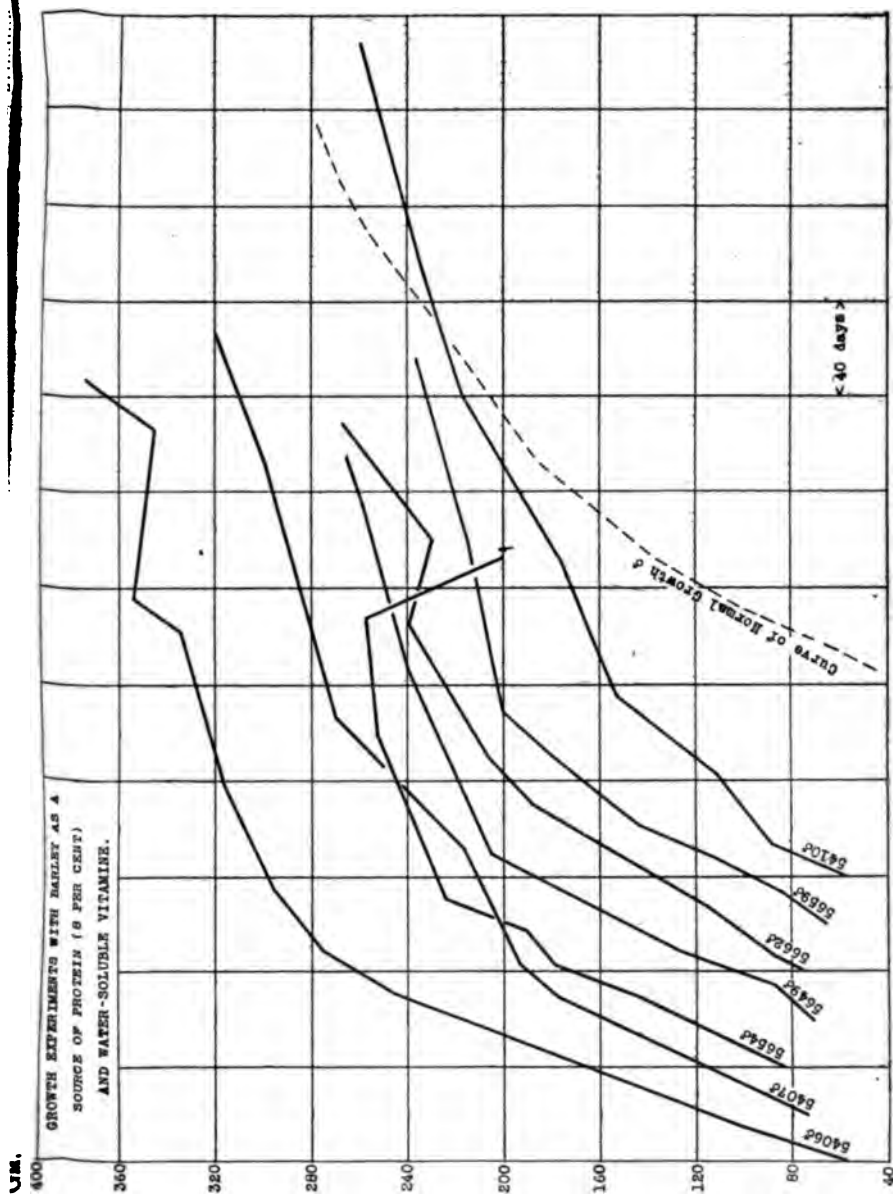


CHART II. Showing the growth of rats on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamins were furnished by barley which formed 72 to 75 per cent of the food mixture

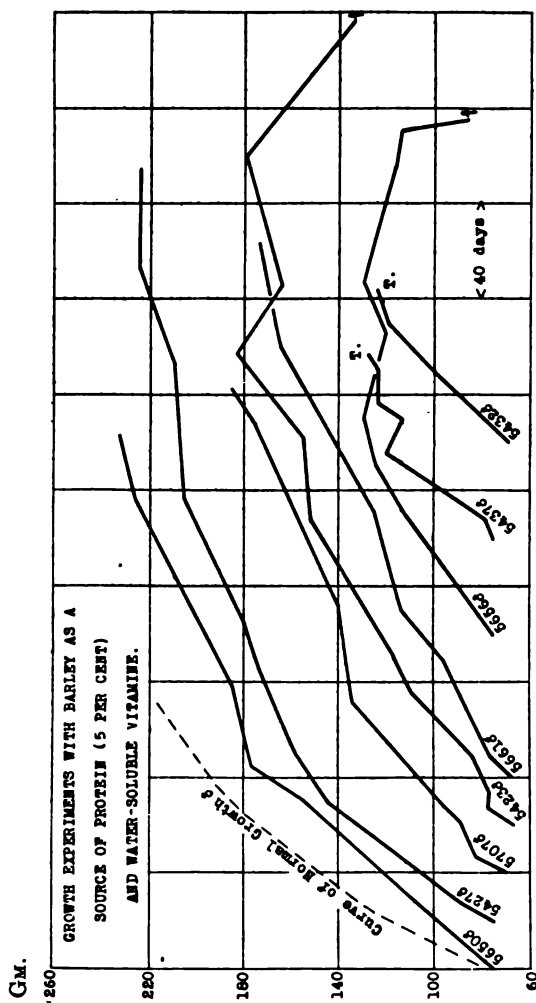


CHART III. Showing the growth of rats on an otherwise adequate diet in which all the protein (5 per cent) and the water-soluble vitamins were furnished by barley, which formed 45 to 47 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests.

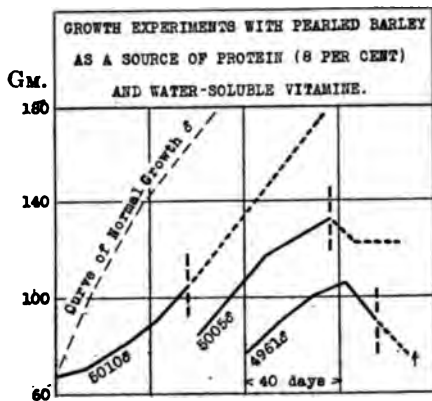


CHART IV. Showing the inferior growth of animals on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamine were furnished by pearled barley, which formed 92 per cent of the food mixture. These results should be contrasted with the curves in Chart II. The failure to grow more vigorously was apparently not due to a lack of water-soluble vitamine in the preparation, since the addition of 0.2 gm. of dried brewery yeast during the period indicated by the interrupted line failed to promote any noteworthy increase in the rate of growth. This quantity of yeast has been demonstrated in repeated comparable experiments to furnish sufficient water-soluble vitamine for growth at a normal rate.

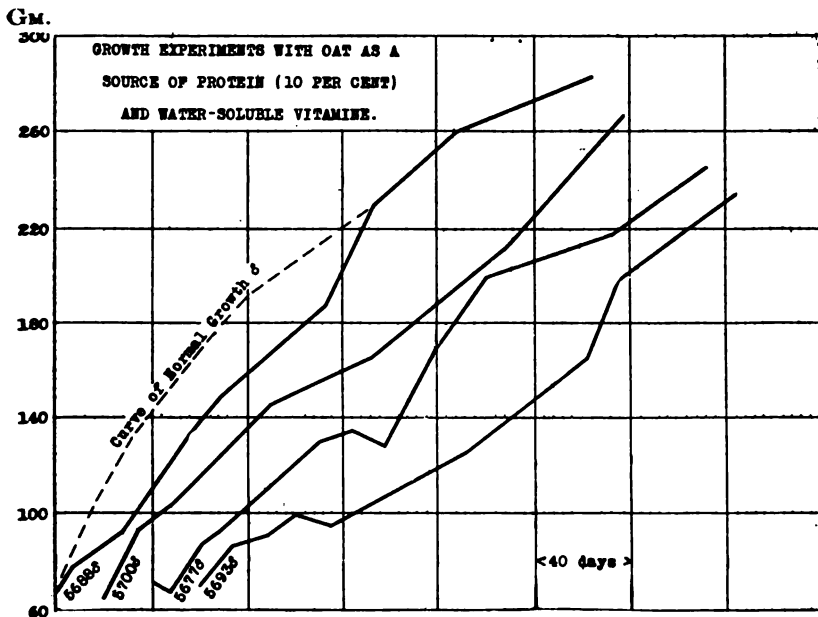


CHART V. Showing the growth of rats on an otherwise adequate diet in which all the protein (10 per cent) and the water-soluble vitamine were furnished by oat kernel, which formed 70 per cent of the food mixture.

CHART VI. Showing the growth of rats on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamins were furnished by oat kernel, which formed 57 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests.

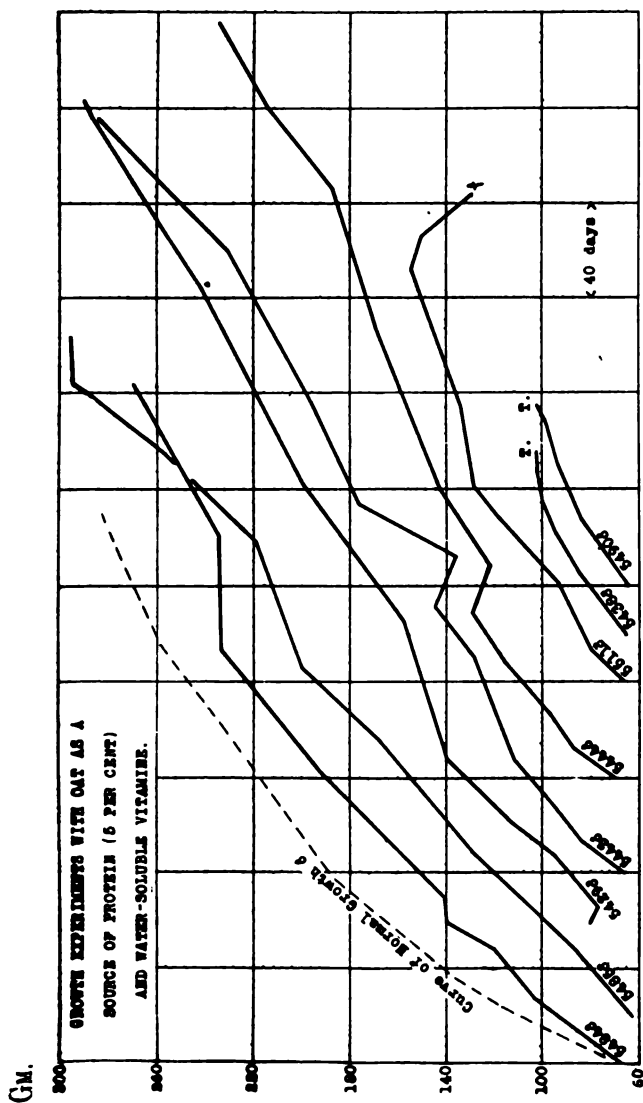


CHART VII. Showing the growth of rats on an otherwise adequate diet in which all the protein (5 per cent) and the water-soluble vitamins were furnished by the oat kernel, which formed 35.5 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests.

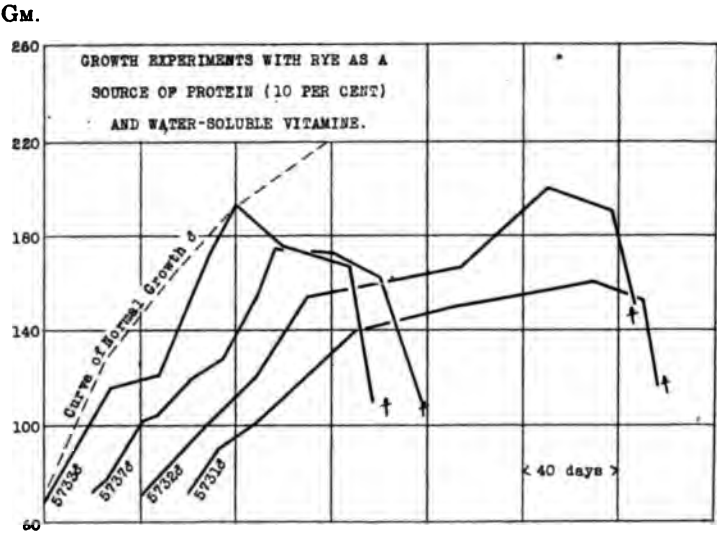


CHART VIII. Showing the growth of rats on an otherwise adequate diet in which all the protein (10 per cent) and the water-soluble vitamins were furnished by whole rye, which formed 80 to 85.4 per cent of the food mixture. The high mortality in the experiment is referred to in the text.

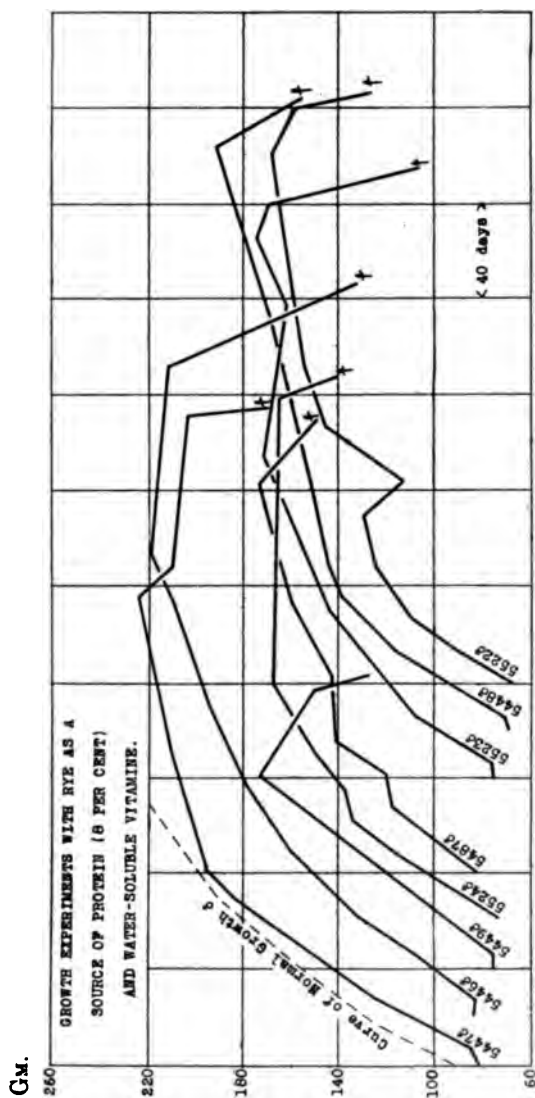


CHART IX. Showing the growth of rats on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamin were furnished by whole rye, which formed 64 to 69.5 per cent of the food mixture. The high mortality in the experiment is referred to in the text.

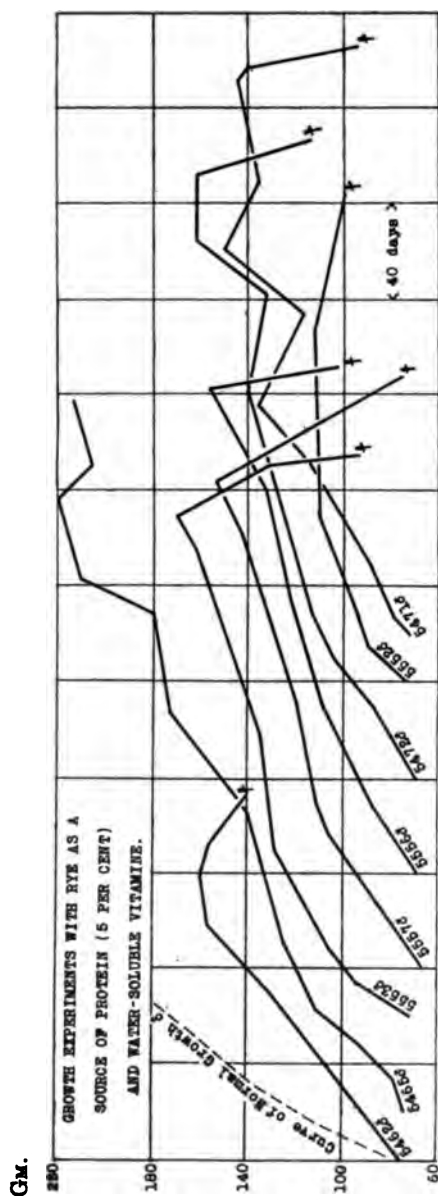


CHART X. Showing the growth of rats on an otherwise adequate diet in which all the protein (5 per cent) and the water-soluble vitamin were furnished by whole rye, which formed 40 to 42.7 per cent of the food mixture. The high mortality in the experiment is referred to in the text.

Gm.

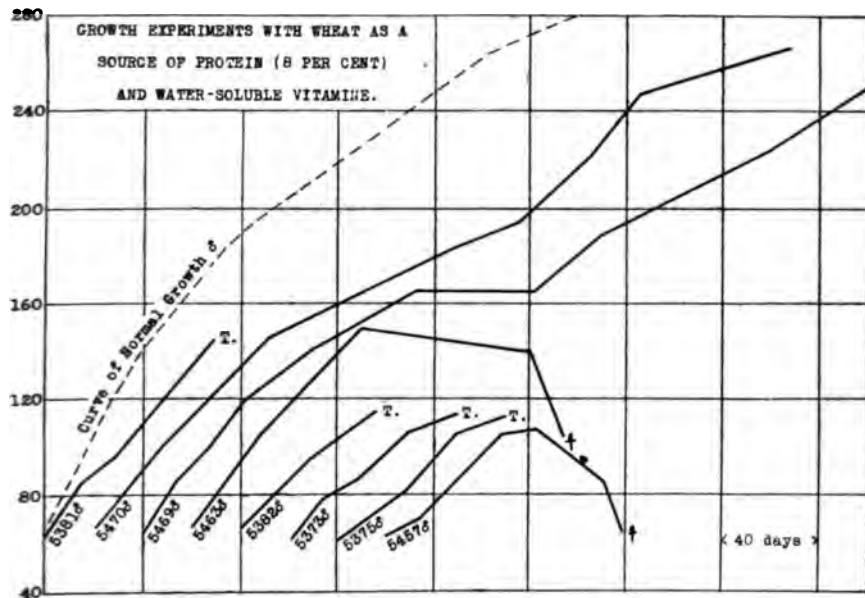


CHART XI. Showing the growth of rats on an otherwise adequate diet in which all the protein (8 per cent) and the water-soluble vitamins were furnished by whole wheat, which formed 70 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests. The normal growth and reproduction of rats on a diet in which the protein and water-soluble vitamins are furnished by whole wheat are indicated by Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557, Chart II.

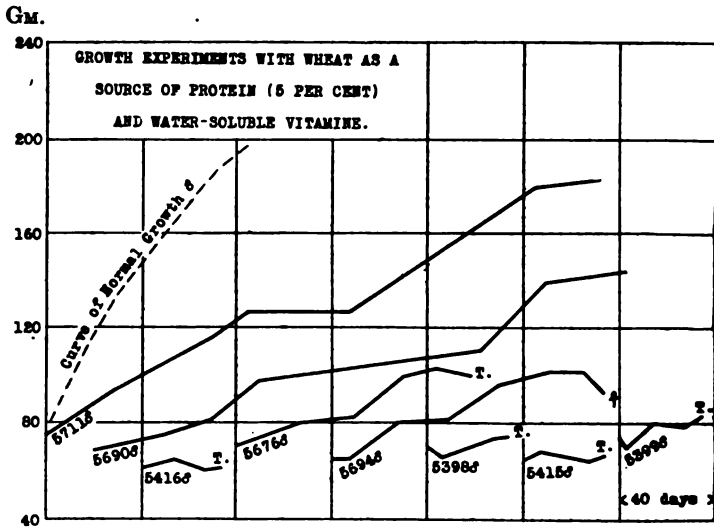


CHART XII. Showing the growth of rats on an otherwise adequate diet in which all the protein (5 per cent) and the water-soluble vitamins were furnished by whole wheat, which formed 51 per cent of the food mixture. Some of the experiments were terminated (T.) early without reference to the final outcome of the tests.

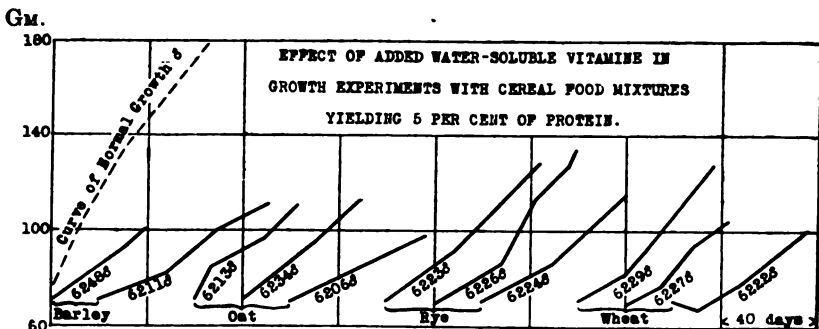


CHART XIII. Showing the effect of daily additions of a protein-free preparation of water-soluble vitamine from yeast, fed apart from the food mixture, which contained 5 per cent of protein furnished by barley, oat, rye, or wheat. The results should be contrasted with those from the comparable experiments on Charts III, VII, X, and XII respectively. They indicate that the failure to grow better on diets containing the smaller proportions of grains, equivalent to only 5 per cent of cereal protein, was not due to the lack of water-soluble vitamine in these instances. The vitamine preparation used is described by Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

EFFECT OF THE CHLORINE SUBSTITUTION PRODUCTS OF METHANE, ACETALDEHYDE, AND OF SODIUM ACETATE ON CATALASE PRODUCTION.*

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(Received for publication, January 13, 1920.)

It is recognized that the introduction of chlorine into the molecule of an aliphatic narcotic greatly enhances its effect as a narcotic. Snow (1), Bert' (2), Arloing (3), Verworn (4), and others have shown that, as a rule, oxidation is decreased during anesthesia and hence the conclusion has been drawn that narcosis may be due to the inhibition or interference with oxidation, while Crile (5) claims that it is due to the acidosis arising from diminished or defective oxidation. We (6) found that narcotics decrease catalase, an enzyme possessing the property of liberating oxygen from hydrogen peroxide, by diminishing its output from the liver and by direct destruction of the enzyme, and hence concluded that the diminished oxidation during narcosis may be due to the decrease in catalase. It was also found that glycocoll, in keeping with Lusk's observation (7) that this amino-acid increased oxidation in the body, produced a very great increase in catalase. Acetic acid or its sodium salt, a substance closely related chemically to glycocoll, was also found to produce a great increase in catalase by stimulating the alimentary glands, particularly the liver, to an increased output of this enzyme.

The present investigation was begun in an attempt to determine whether the introduction of increasing amounts of chlorine into the methane molecule would increase its destructive effect on catalase, and whether the introduction of increasing amounts

* An abstract of this paper was read at the annual meeting of the Federation of American Societies for Experimental Biology, Cincinnati, December, 1919.

of chlorine into the acetic acid molecule would diminish its effectiveness in producing an increase in catalase. The animals used were dogs, rabbits, and cats. The substances used were methane (CH_4), monochloromethane (CH_3Cl), dichloromethane (CH_2Cl_2), trichloromethane (chloroform, CHCl_3), tetrachloromethane (CCl_4); acetaldehyde (CH_3CHO), chloral (CCl_3CHO); sodium monochloroacetate ($\text{CH}_2\text{ClCOONa}$), sodium dichloroacetate ($\text{CHCl}_2\text{COONa}$), and sodium trichloroacetate (CCl_3COONa). The amounts of the substances will be given in the description of the experiments. The catalase was determined by adding 0.5 cc. of blood to diluted hydrogen peroxide at approximately 22°C . in a bottle, and the amount of oxygen gas liberated in 10 minutes was taken as a measure of the amount of catalase in the 0.5 cc. of blood. Account was taken in these experiments and corrections were made for the inhibiting action of acids and acid salts on catalase as observed by Jacobson (8), Loevenhart and Kastle (9), Issajew (10), Senter (11), Winternitz and Rogers (12), Mendel and Leavenworth (13), and Bodansky (14), as well as the effect of alkalies and alkaline salts.

In Fig. 1 are shown the effects of methane, acetaldehyde, and sodium acetate together with that of their chlorine substitution products on the blood catalase *in vivo* as well as *in vitro*. The figures along the abscissæ indicate time in minutes and the figures along the ordinate, percentage increase or decrease in catalase. The trichloromethane or chloroform, and tetrachloromethane were administered by bubbling air through these substances in a bottle which was connected by a rubber tube to a cone adjusted over the snout of the animal, while the methane, mono- and dichloromethane were led directly from the generator into an inverted glass vessel containing the animal. The animals used were rabbits. The catalase in 0.5 cc. of blood from the jugular vein was determined before as well as at intervals after the administration of the substances. In the chart it may be seen that methane (CH_4) had little or no effect on the blood catalase; monochloromethane (CH_3Cl) decreased it 22 per cent in 45 minutes; trichloromethane (chloroform, CHCl_3), 32 per cent; and tetrachloromethane (CCl_4), 37 per cent. From these figures it may be seen that the more chlorine there is in the methane molecule, the more effective it becomes in decreasing the blood catalase.

It may be seen further in the chart that glycocoll ($\text{CH}_2\text{NH}_2\text{COOH}$) increased the blood catalase 52 per cent in 60 minutes; sodium acetate (CH_3COONa), 42 per cent; sodium monochloroacetate ($\text{CH}_2\text{ClCOONa}$), 37 per cent; sodium dichloroacetate ($\text{CHCl}_2\text{COONa}$), 23 per cent; and sodium trichloroacetate (CCl_3COONa) produced practically no change in the blood catalase. Cats were used in these experiments. The amounts of the substances used were 10 gm. per kilo dissolved in 75 cc. of water. The substances were introduced into the upper part of the small intestine and the blood from the jugular vein was used for the catalase determinations. It may also be seen that 0.6 gm. per kilo of acetaldehyde (CH_3CHO) decreased the catalase 12 per cent, and that a similar amount per kilo of its chlorine substitution product, chloral (CCl_3CHO), decreased the catalase 22 per cent in 90 minutes. The animals used in these experiments were rabbits and the substances, dissolved in 50 cc. of water were introduced by means of a stomach tube.

The second part of this paper is concerned with determining the mode of action of the chlorine substitution products of methane and acetaldehyde in producing a decrease and of sodium acetate in producing an increase in catalase and also in finding an explanation for the fact that the introduction of chlorine into the acetic acid molecule diminishes its effectiveness in increasing catalase. In Fig. 1 under "*in vitro*" are shown the effects on the catalase of cat's blood when exposed to methane and its chlorine substitution products. The amount of defibrinated blood used was 5 cc., which was poured into a glass vessel in which it formed a layer about 2 mm. thick. Such preparations were exposed to methane and to its chlorine substitution products at 40°C . for the times indicated in the chart. It may be seen that the exposure of the blood to methane had practically no effect on its catalase; the exposure to monochloromethane gas decreased the catalase very little in 60 minutes; the exposure to dichloromethane decreased it 21 per cent in 15 minutes and 62 per cent in 45 minutes; the exposure to trichloromethane gas decreased it 48 per cent in 15 minutes and 76 per cent in 45 minutes. From these figures it is clear that increasing the amount of chlorine in methane increased its destructive effect on catalase *in vitro*. It may be seen further in the chart that neither sodium acetate nor any of its chlorine sub-

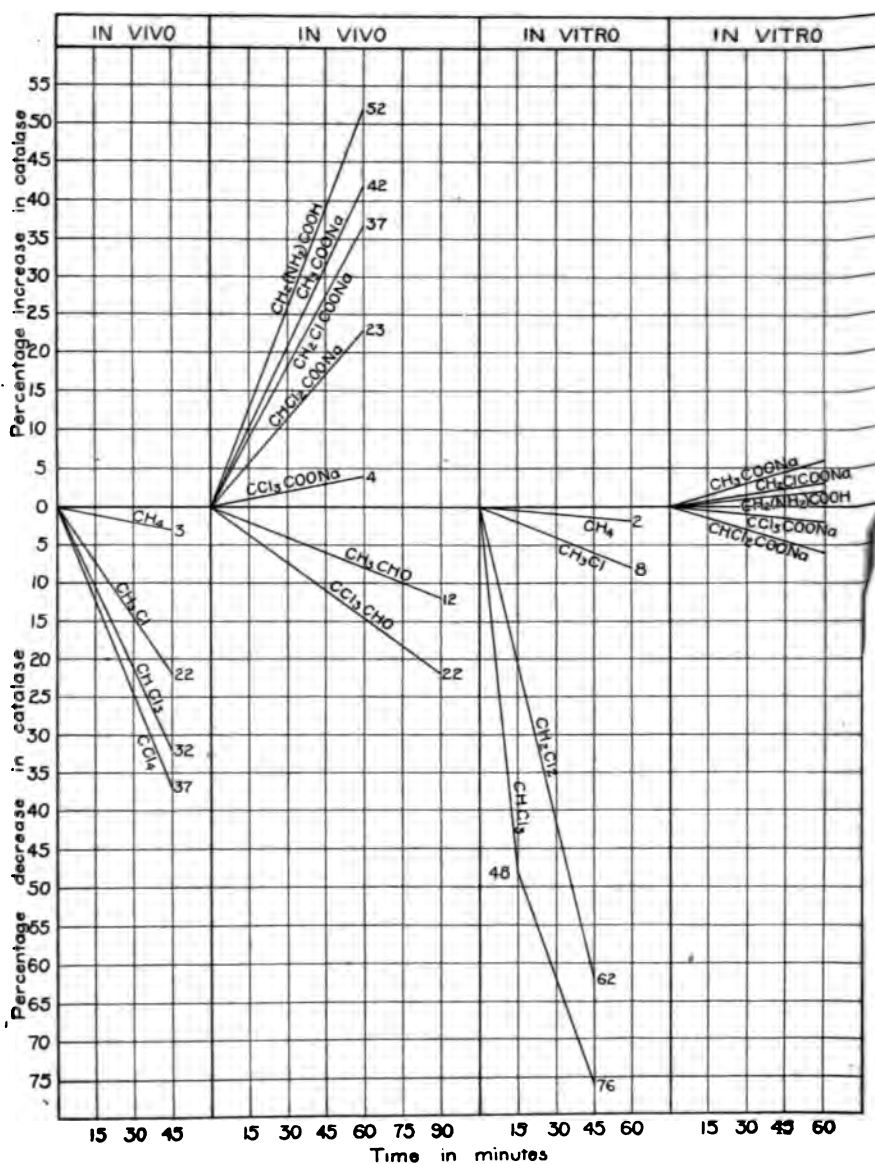


FIG. 1. Curves showing effect of the substances named in the chart on the catalase content of the blood.

stitution products had any effect on catalase *in vitro*. 100 mg. of each of these substances were added to 5 cc. of defibrinated cat's blood and the catalase determinations, using neutral hydrogen peroxide, were made at the intervals indicated in the chart.

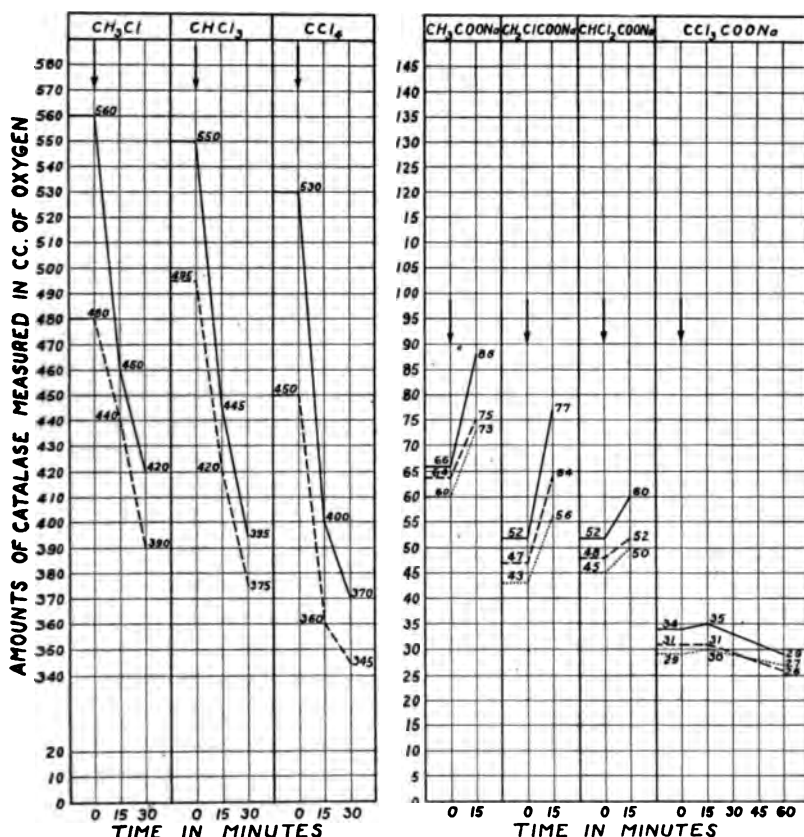


FIG. 2. Curves showing effect of the substances named in the chart on the catalase content of the blood. The continuous line curves show amount of catalase in the blood of the liver, the dash line curves the amount in the blood of the portal vein, and the dotted line curves the amount in the blood of the jugular vein.

In Fig. 2 are shown the effects of the administration of the chlorine substitution products of methane and of sodium acetate on the catalase of the blood of the liver, portal, and jugular veins.

The continuous line curves were constructed from data obtained from the blood of the liver, the dash line curves from the blood of the portal, and the dotted line curves from the blood of the jugular vein. The chloroform and tetrachloromethane were administered by bubbling air through these substances in a bottle which was connected by a rubber tube to a cone adjusted over the snout of the animal while the monochloromethane was led directly from the generator to the animal. These substances were administered in as concentrated form and in as large amounts as could be done and at the same time keep the animal alive. The animals used for the study of these chlorine substitution products were cats. It may be seen under monochloromethane CH_3Cl that previous to the use of this material 0.5 cc. of blood from the liver liberated 560 cc. of oxygen from hydrogen peroxide in 10 minutes and that 0.5 cc. of blood from the portal vein of the same animal liberated 480 cc.; after 15 minutes use of the monochloromethane the blood of the liver liberated 460 cc. of oxygen and that of the portal vein 440 cc. and after 30 minutes the blood of the liver liberated 420 cc. of oxygen and that of the portal vein 390 cc. Under trichloromethane CHCl_3 and tetrachloromethane CCl_4 ; it may be seen that these substances also produced a decrease in the catalase of the blood of the liver and of the jugular vein. By comparing these figures it is evident that the catalase content of the blood of the liver in all the animals used was much greater than that of the blood of the portal vein. This is taken to mean that the liver is putting out catalase continuously into the blood. By comparing the decreases produced by these substances in the catalase of the blood of the liver and of the portal vein it may be seen that they produced a much greater decrease in the blood of the liver than they did in the blood of the portal vein. This is taken to mean that these substances were decreasing the output of catalase from the liver. It should be mentioned in this connection that Becht (15) has repeated some of our work on the effect of narcotics on the blood catalase and claims that catalase is increased instead of being decreased during narcosis.

In Fig. 2 are shown also the effects of sodium acetate and its chlorine substitution products on the catalase of the blood of the liver, portal, and jugular veins. The animals used were dogs. After opening the abdominal wall of these animals with the use

of ether anesthesia, 10 gm. per kilo in 300 cc. of water were introduced into the upper part of the small intestine. By comparing these figures it may be seen that sodium acetate produced an increase in the catalase of the blood and that this increase was greater in the blood of the liver than it was in the blood of the portal and jugular veins. This suggests that the sodium acetate was stimulating the liver to an increased output of catalase. Similarly it may be seen that the mono- and dichlorine substitution products of sodium acetate produced an increase in catalase in 15 minutes while the trichlorine substitution product produced a small decrease in 60 minutes. By comparing these figures it may be seen that the effect of the introduction of chlorine into the sodium acetate molecule was to decrease its effectiveness in stimulating the liver to an increased output of catalase.

SUMMARY.

1. The more chlorine that is introduced into the methane molecule the more effective it becomes in decreasing catalase. Similarly, the strong narcotic chloral, a trichlorine substitution product of acetaldehyde, decreases catalase more than does acetaldehyde, a weaker narcotic. The ingestion of sodium acetate produces an increase in catalase. The introduction of increasing amounts of chlorine into the sodium acetate molecule renders it less effective in increasing catalase.

2. The chlorine substitution products of methane decrease catalase by direct destruction of the enzyme and by decreasing its output from the liver. Sodium acetate produces an increase in catalase by stimulating the liver to an increased output. The introduction of chlorine into the molecule renders it less effective as a stimulant on catalase production.

3. The increase in oxidation following the ingestion of glycocholl or a closely related compound, acetic acid (sodium salt), is attributed to the increase in catalase. The decrease in oxidation arising during chloroform and chloral anesthesia and assumed by some to be the cause of the narcosis is attributed to the decrease in catalase.

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AN IMPROVED VOLUMETRIC PUMP FOR CONTINUOUS INTRAVENOUS INJECTIONS.

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Plate 3.

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A machine described earlier¹ consisted essentially of a single glass syringe fitted with a two-way valve, syringe and valve being operated by an electric motor acting through a worm gear, eccentrics, and rods. The discharge of the pump was controlled coarsely by setting the stroke of the piston by means of a system of levers and more exactly through control of the motor speed by means of a rheostat. This system involved the use of variable speed motors and changing motor speeds, with the inherent disadvantages which this implies from the standpoint of uniform performance.

The present machine mounts two glass syringes or cylinders each fitted with a two-way valve. Both pumps are run by one motor acting as before through a worm gear, eccentrics, etc. The former method of adjusting the stroke is displaced by a new device which is applied separately to each piston rod. The new device is simpler and much more accurate than the old and permits the stroke of either piston to be set independently in a few seconds at the desired length while the machine is running or stopped. Owing to the stroke adjustment it becomes unnecessary to alter the speed of the motor during an experiment, thus making it possible to drop the variable speed motor and rheostat in favor of a motor of constant speed type with the decided advantage that the operator's attention is not required to secure uniform performances. With a half-horse-power "Synchronous" motor, long experiments involving repeated changes in the rate of dis-

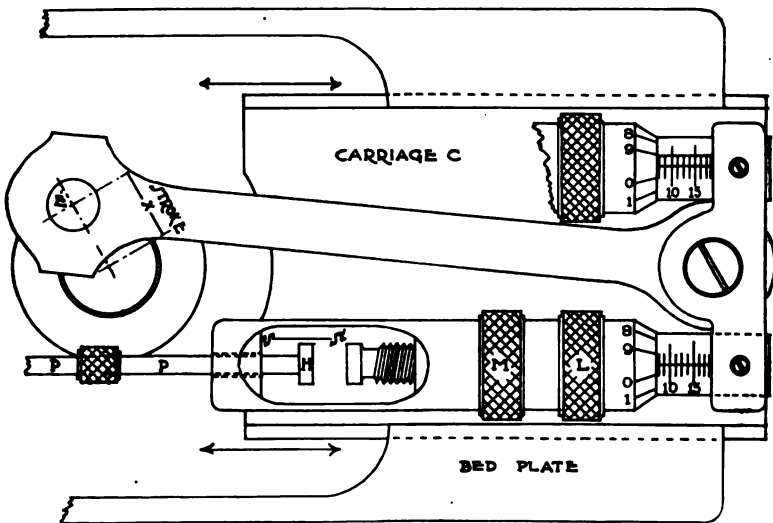
¹ Woodyatt, R. T., *J. Biol. Chem.*, 1917, xxix, 355.

charge from one or both cylinders have been conducted easily and with negligible fluctuations of the motor speed.

The stroke adjustment is illustrated in Text-fig. 1. The motion of the motor is transmitted through a worm gear to the eccentric E and by the eccentric rod to a carriage plate C. The carriage C moves in a grooved bed plate in the direction of the arrows making a stroke equal to the stroke X of the eccentric. The diagram shows the piston rod P broken off at its point of emergence from the pump cylinder. The piston rod is jointed for convenience in taking the machine apart and terminates in a hard steel head H standing free in the space SS'. This space is the measuring gap in a machinist's micrometer modified for the purpose. The micrometer is fixed by a mounting on the right to the carriage itself and projects to the left over the plane of the carriage with clearance to permit of its operation. The distance SS' can be given any value desired by loosening the lock nut L and turning the milled collar M which causes the surface S to move toward or away from S'. When the machine is in motion it will be seen that the distance SS' can be made so great that after one complete stroke the head H will have been pushed as far to the left as the extreme forward motion of S' will permit, after which it will remain stationary, S' just touching it at the end of each subsequent forward stroke while on the back stroke S will merely approach H. But as the gap SS' is shortened by turning M so that S approaches S' a position may be found in which at the end of the back stroke S will also just touch H. With this setting of the micrometer, H is just touched but not moved on the forward stroke by S' and on the back stroke by S. This position corresponds to 0 on the scale and collar of the micrometer. The carriage now moves back and forth through the distance X while none of its motion is imparted to the piston although while the piston remains stationary the valves are turned. Now if S is set 1 cm. closer to S' as read directly on the scale and collar, then H will be displaced 1 cm. on the back stroke and on the forward stroke will return to its former position, and so on for any setting within the range of the apparatus. The micrometer reads to 0.04 mm.

It will be noted that with this device the head H and so the piston rod and piston are free during the interval after the surface

S' having pushed H to the limit is receding and while the surface S is approaching H but is not yet in contact with it. This interval follows immediately upon the completion of the systole of the pump when the pressure of the fluid in the discharge tube leading from the pump is highest. If sufficient back pressure develops it may kick back the piston before the valve cuts off the communication thus destroying the quantitative character of the pump and reducing the total discharge. There is a similar interval at the end of the diastole during which the pressure of the gravity feed



TEXT-FIG. 1. Improved volumetric pump for continuous intravenous injections. The actual length is 10 inches over all.

if such is used might move a loose piston and allow the cylinder to take in more than the indicated volume of fluid at each stroke. These sources of error are eliminated by a friction check on the piston rod which makes it impossible for the piston to move except in response to impacts of the surface S and S'.

The present machine has the advantages of two single machines of the earlier type in that it permits the simultaneous injection of one or two liquids into one or two discharge tubes, both constantly at different rates bearing known ratios, one constantly

at one rate with one varying, or both varying as desired. The accuracy and evenness of performance as well as the convenience of operation are much higher than in the older machine. Having a motor which runs always at the same speed, a cylinder (syringe) is calibrated by direct observation of the total volume which it discharges during periods of 15 minutes to 1 hour, with the stroke set at 0, at 15 mm., and at two or more intermediate points. In plotting the results they are found to fall on a straight line and the chart so formed indicates the setting of the micrometer necessary for the delivery of any desired volume per hour. When set to deliver a given volume in an hour, the results with the present motor fed from an ordinary power circuit have frequently fallen within 0.1 to 0.3 cc. of the desired total.

The machine was made by William Gaertner and Company, 5345 Lake Park Avenue, Chicago, Illinois, and is illustrated in Fig. 1.

EXPLANATION OF PLATE 3.

FIG. 1. Assembled machine. The syringes and valves are demountable without tools for cleansing and sterilization purposes. The large screw head at the left and below is at the end of the worm shaft bearing. The motor shaft is coupled to the opposite end of the worm shaft.



FIG. 1.

(Woodyatt: Pump for intravenous injections.)



BIOCHEMISTRY OF THE ACETONE AND BUTYL ALCOHOL FERMENTATION OF STARCH BY *BACILLUS GRANULOBACTER PECTINOVORUM*.

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(Received for publication, January 10, 1920.)

During the last 4 years a considerable amount of research, chiefly along industrial lines, has been performed in connection with the production of acetone together with various alcohols by the fermentation of carbohydrate media, using cultures of different bacteria. Some of this work has already been described in the literature (1, 2). Contributions have also been made to our knowledge of the biochemistry of these important processes. Northrop and his collaborators (3) have studied in detail the behavior of *Bacillus acetoethylicum* in standard media, and also the influence of certain factors, namely the reaction of the media, the size of the inoculation, the supply of air and nitrogen (peptone), and the temperature of the medium, on the growth of the organism and the yields of acetone and ethyl alcohol. They record that the principal acid produced during the fermentation is formic. At a conference held in London during the past year the position regarding the production of acetone with butyl alcohol by fermentation methods was discussed (4). More recently Fred and his collaborators (5) have shown that during the fermentation of xylose a mixture of acetic and lactic acids is produced, using cultures of bacteria isolated from manure, silage, etc.

An effort has been made during this investigation to identify and study the rôle of the intermediate compounds formed during the fermentation of starch by *Bacillus granulobacter pectinovorum*.¹

¹ The culture used in the research has been developed from tubes received from Dr. C. Weizman in 1915 when it was my privilege to be associated with him in the early stages of the more general investigation.

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Products of the Fermentation.

At the meeting in London the percentage yields of acetone and butyl alcohol were given as 7 and 14 per cent respectively of the weight of maize meal used. During the last 3 years very large volumes of these two compounds were prepared by this process in Toronto, and by an analysis of the data obtained we were able to calculate the various yields. The following is a summary of these calculations.

Weight of dry corn distilled.....	73,463,654 lbs.
Gross production of acetone.....	6,248,131 "
Net " " "	5,741,273 "
Total " " alcohols.....	12,660,834 "
Gross yield of acetone on dry corn distilled.....	8.5 per cent.
Net " " " " " " "	7.76 " "
Yield of alcohols on dry corn distilled.....	17.23 " "

These results indicate that as the culture and methods of working have improved the percentage yields have increased.

During the distillation it was discovered that ethyl alcohol occurs in the middle fractions. In addition to the large low and high boiling fractions, containing acetone and butyl alcohol respectively, a large fraction is obtained boiling between 85–91.5°C. The latter contains the three neutral products of the fermentation and water. By redistillation pure ethyl alcohol, boiling point 78°C., has been prepared from it. The chemical staff engaged in the work has estimated that the total weight of alcohol produced contains approximately 7.6 per cent of ethyl alcohol.

During the fermentation large volumes of carbon dioxide and hydrogen are produced. The sugar and organic acids formed during the fermentation have been isolated and identified. An attempt has also been made to show the part played by these compounds in the biochemical system underlying the production of the two alcohols and acetone.

Hydrolysis of Starch.

The medium used was in the form of a starch gel, with a heavier layer at the base composed of fiber and protein-containing tissue. Immediately after inoculation liquefaction of the starch com-

mences. By the usual methods it was found that with the liquefaction there is a rapid formation of a reducing sugar. If a small volume of the clear fluid is removed from the flask and added to a 1 per cent starch paste with a slight amount of toluene, it is found that after 24 hours the starch is completely hydrolyzed to a sugar which is a strong reducer of Fehling's solution and forms an osazone very rapidly. From these results and those from suitable controls it was concluded that an enzyme is secreted by the organism which acts upon the starch gel.

The osazone produced was isolated and purified. Only crystals having the characteristic form of glucosazone were to be seen in the specimens obtained from many experiments of this nature. The crystals were found to melt at 204.5°C.

Larger volumes of mash were then inoculated and at the end of 4 hours the flasks were removed from the incubator and an excess of toluene was added to the contents. These were dialyzed against distilled H₂O or Ringer's fluid for 6 days. A mixture of sugar and protein cleavage products passed through the membrane. The solution was evaporated to a small volume under reduced pressure and decolorized with animal charcoal. The residue was extracted with 95 per cent alcohol and reconcentrated. The 95 per cent alcohol solution was then allowed to evaporate at 35°C. A mass of needle-shaped crystals was obtained. The aqueous solution of these crystals was dextro-rotatory. From these facts we conclude that the first stage in the fermentation is the hydrolysis of starch to glucose. The nature and properties of the enzyme responsible for this conversion are being more fully investigated.

Acidity Changes.

To obtain information regarding the acidity changes in the fermenting mash, samples of the medium uniform in volume were titrated at regular intervals with 0.1 N NaOH. Curve A in Chart 1 expresses such changes in a normal fermentation. By means of these results the fermentation period can be divided into three sections: (a) the time during which the acidity is rising to a maximum, followed by (b) the time during which the acidity is falling, and (c) a third period during which the acidity rises very slowly from the minimum reached in (b).

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Curve B in the same chart shows the rate of gas production of this fermentation, based on hourly readings of the rate. We find that during the first of the three phases of the fermentation period the rate rises steadily. When the acidity of the mash is approaching and at the maximum, the rate of gas production falls

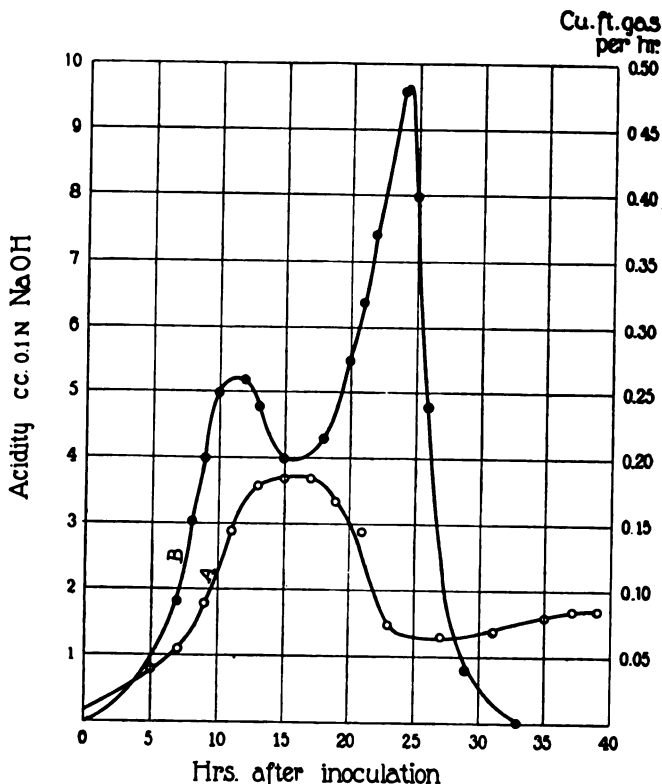


CHART 1. Curve A represents the acidity of 10 cc. samples in terms of 0.1 N NaOH; Curve B the gas produced per hour during the fermentation.

temporarily, and during the second phase rises once more very rapidly to a maximum. Towards the close of the fermentation the rate falls sharply to zero.

The acidity changes and gas production have been studied in two types of abnormal fermentations. For reasons which are

often difficult to define, the life cycle period of the bacillus is sometimes much prolonged. The fermentation is sluggish and the yields are poor. In addition to the morphological features of the organism, such a fermentation has characteristic acidity and gas rate curves. In Chart 2 examples are shown. Curves A and B represent the acidity and rate of gas production readings of a

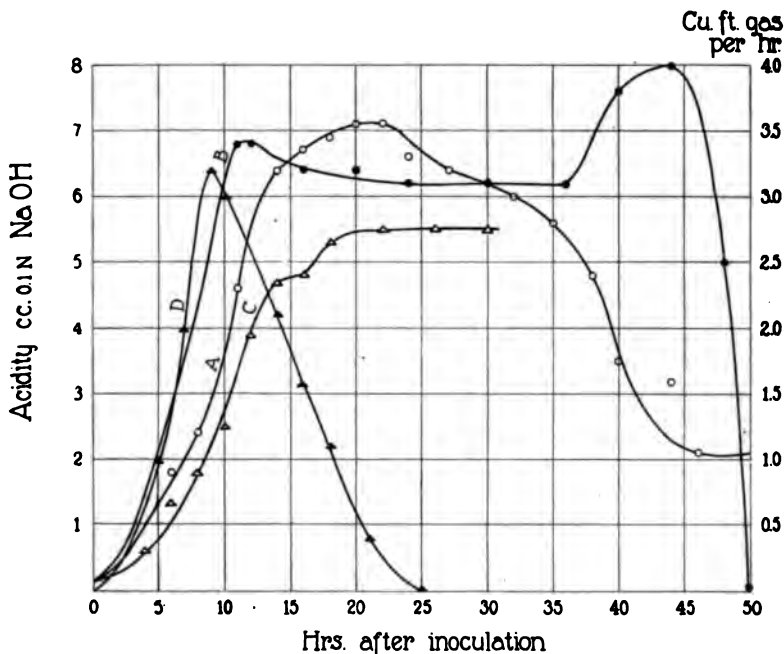


CHART 2. Curve A is the acidity curve and Curve B the gas curve of a slow fermentation. Curves C and D are the corresponding curves for a fermentation conducted at 110° F.

fermentation, volume 20,000 gallons, which was inoculated on September 28, 1917.

With the abnormal prolongation of the maximum acidity period, there is a long delay in the occurrence of the rapid rise in the rate of gas production, and when this increase does develop the maximum rate is very much below the normal.

In the same chart Curves C and D relate to an experiment in which the fermentation was conducted at 110° F. instead of 98° F.

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Experiment 1.—A mixture of 3.5 gallons of water and 2 pounds of meal was made in a copper culture vessel. The vessel was maintained at 7 to 8 pounds steam pressure for 3 hours, and the contents were then cooled to the temperature required. To the mash 300 cc. of an active culture were then added. The acidity was determined at regular intervals and the gas produced measured on a small experimental gas-meter. The results obtained are presented in Table I.

TABLE I.

Date.	Time after inoculation.	Gas per hr.	0.1 N NaOH required for 10 cc. sample.
1917	hrs.	cu. ft.	cc.
Nov. 30, 2.00 p.m.....			
6.00 "	4	0.140	0.9
8.00 "	6	0.290	
12.00 m.	10	0.445	2.4
Dec. 1, 2.00 a.m.....	12	0.379	3.9
4.00 "	14	0.320	4.6
6.00 "	16	0.244	5.1
8.00 "	18	0.161	5.1
12.00 n.	22	0.026	5.0
2.00 p.m.....	24	0.012	5.5
4.00 "	26	0.007	5.4
6.00 "	28	0.004	5.6
8.00 "	30	0.002	5.4
10.00 "	32		5.4

We find that working under the conditions mentioned, it is possible to obtain the following results: (a) the rate of gas production does not rise after the preliminary fall which has already been observed in the normal, but continues to fall to 0 and (b) the acidity does not fall from the normal maximum, but after a slight pause a second increase occurs, and finally an abnormal and constant maximum is reached.

These examples of three different types of fermentation indicate that in the biochemical system there is a close association between the activity of the living cell, expressed by the evolution of gas, and the changes in the acidity of the medium. We may conclude that the fall in acidity during the second phase of the fermentation period is dependent upon organized cell life processes and is not the result of free enzyme activity.

Influence of Starch Concentration on the Acidity Changes of the Medium.

The following are examples of a series of experiments which was carried out to determine in what way the acidity of the fermenting mash was influenced by increasing the concentration of solids. The concentrations used were within the range in which the rate of fermentation is proportional to the concentration of starch in the medium.

Experiment 2.—Three flasks containing maize mash of the concentrations 3, 5, and 7 per cent were sterilized in the autoclave for 2.5 hours at 10 pounds steam pressure. They were inoculated with test-tube cultures of the bacillus in 5 per cent mash. The acidity of the flasks was determined at regular intervals, and the results from the experiment are given in Table II.

TABLE II.

Meal.	Acidity after inoculation. 0.1 N NaOH required for 10 cc. sample.						
	14.5 hrs.	16.5 hrs.	19.5 hrs.	21.5 hrs.	26.5 hrs.	38.5 hrs.	43.5 hrs.
per cent	cc.	cc.	cc.	cc.	cc.	cc.	cc.
3	3.20	3.90	4.20	4.10	2.60	2.20	2.00
5	3.95	4.95	5.80	5.90	5.00	1.90	2.30
7	4.00	5.60	6.60	6.60	5.70	1.95	2.30

Experiment 3.—The last experiment was repeated with the exception that the flasks fermented contained 2, 4, 6, and 8 per cent mash respectively. In connection with these experiments it is necessary to emphasize the importance of a correct choice of culture with which to inoculate the flasks. It must all be derived from the same spore stock, and the tubes, containing equal volumes, must all be of the same generation and the same age. The results from this experiment are given in Table III.

TABLE III.

Meal.	Acidity after inoculation. 0.1 N NaOH required for 10 cc. sample.							
	7 hrs.	12 hrs.	15 hrs.	20 hrs.	24 hrs.	34 hrs.	40.5 hrs.	60 hrs.
per cent	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
2	0.9	1.8	2.2	2.8	2.7	1.8	1.8	2.0
4	1.2	2.7	3.5	4.2	3.6	2.0	2.0	
6	1.5	3.3	4.5	4.8	4.5	3.1	1.8	
8	1.9	3.6	5.0	5.3	4.9	2.6	1.9	

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These results indicate that the acidity of the mash during the fermentation is controlled at least in part by the concentration of meal used. We find that in each series of flasks the acidity increased with the concentration. The two series of flasks when grouped together do not form a regular sequence as regards both the concentration of the mash and the acidity readings. For example, the 3 per cent flask of the first set does not occupy a position between the 2 and 4 per cent flasks of the second set. This condition of things has been confirmed during a much wider study of fermentations of various concentrations. It is clear that the influence of the concentration of starch in the medium on the acidity must be regarded as one only of several factors affecting the latter.

Influence of the Rate of Fermentation on the Maximum Acidity.

Until the biochemistry of the fermentation is more completely analyzed and understood we can only group together a number of these unknown factors and express the sum of their influences in general terms, such as the time occupied by the fermentation of a known weight of meal, or the yields of the various products under certain conditions. In the production of acetone on a large scale, when the time factor was of great importance, we expected those fermentations which had a maximum acidity higher than the average to occupy a longer time period; those with a lower maximum to be correspondingly quicker. To verify such a hypothesis the accumulated observations of a large number of carefully controlled experiments will be required. At present there are available the laboratory records of several thousands of fermentations, showing the volume and concentration of the medium, the time occupied by, the gas produced by, and the acidity changes of the fermentation. They were obtained originally for practical purposes, but although not free from errors they may assist in the consideration of this problem. One of my colleagues has taken from the records several hundreds of complete sets of observations relating to large fermentations of uniform volume and concentration. These have been arranged in groups according to the number of hours in the fermentation period. The number of examples in each and the average maximum acidity

TABLE IV.

Time of fermentation.	No. of examples.	Average maximum acidity.	Time of fermentation.	No. of examples.	Average maximum acidity.
<i>Hrs.</i>		<i>cc.</i>	<i>Hrs.</i>		<i>cc.</i>
24	9	5.1	35	35	5.8
25	16	5.5	36	36	5.65
26	34	5.06	37	30	5.87
27	35	5.3	38	28	5.9
28	70	5.46	39	13	5.6
29	92	5.5	40	18	5.9
30	81	5.46	41	16	5.9
31	68	5.7	42	10	6.1
32	59	5.6	43	7	6.2
33	60	5.7	44	3	6.3
34	54	5.76			

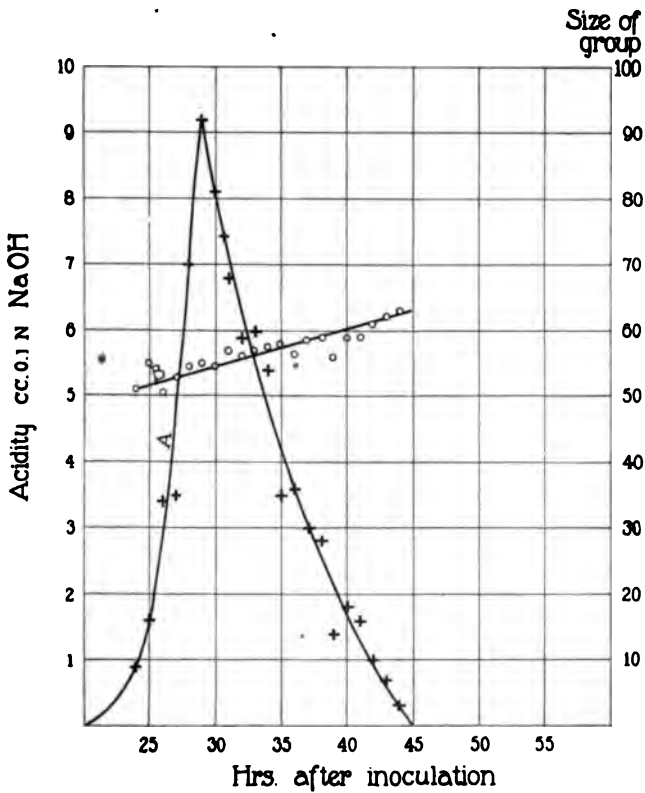


CHART 3. The curves are based on the figures contained in Table IV.

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of the groups were then calculated. These results are given in Table IV. The acidity readings are for 10 cc. samples in terms of 0.1 N NaOH.

These results are represented by curves in Chart 3. Curve B shows the maximum acidity readings, and furnishes evidence in support of the view that the rate of the fermentation and the acidity of the medium are correlated. Curve A represents the number of examples in each group, and is thus a normal distribution curve of fermentations for the time occupied. These figures were calculated for the period from the time of inoculation to the end of fermentation. For a period of approximately 8 hours the filling of the tank was in progress, and therefore these figures cannot be compared directly with those relating to fermentations of a known volume of medium, inoculated at a given time and afterwards allowed to ferment undisturbed.

Isolation and Identification of Volatile Organic Acids.

The following is a description of the methods used on three occasions when the acids have been obtained and identified. Batches of 50 gallons of mash were prepared, sterilized, and fermented in a copper vessel in the laboratory. During the early stages of the fermentation the acidity of the mash was determined at regular intervals. When successive readings indicated that the acidity was approaching the maximum, the vessel was connected to an adjacent water-cooled, coil condenser. The contents were quickly raised to boiling point, and about 13 gallons of distillate collected in large glass vessels. The whole distillate was saturated with common salt, and extracted with 1,500 cc. of ether. After separating, the ether-soluble fraction was filtered and the ether partially removed by distillation from a water bath maintained below 40°C. The mixture of neutral and acid products of the fermentation was diluted with an equal volume of distilled water. The amount of acid present was estimated in terms of butyric acid and the mixture was then completely neutralized with twice the required amount of calcium carbonate. After an interval of several hours the excess carbonate was removed by filtration under pressure, and was then washed with distilled water. The combined solutions of calcium salts were

evaporated to dryness on the steam bath, and the residues obtained were more thoroughly freed from traces of the neutral compounds by dry heat in the oven, maintained at 100°C. The dry salts were weighed and the amount of sulfuric acid required to liberate the organic acids was calculated. An excess of dilute sulfuric was then added to the salts. The mixture was extracted with two batches of ether, and the united portions of ether-soluble material were distilled in the following manner.

The ether was removed on the water bath maintained at temperatures below 40°C. The fractions of the remainder which came over below 130°C. were removed at atmospheric pressure, and the residue was distilled under reduced pressure. The following results are from the records of these experiments.

Experiment 4.—

	A.	B.
Calcium salts obtained.....	150 gm.	112 gm.
Mixture of acids distilled.....	80.00 cc.	88.00 cc.

Distillation 1.

Fraction:

	A. cc.	B. cc.
(a) Below 40°C.....	0.00	15.3
(b) 40–70°C.....	0.00	0.00
(c) 70–105°C.....	0.00	1.40
(d) 105–130°C.....	11.00	12.70
(e) Above 130°C.....	67.00	55.00

Practically the whole 105–130°C. fraction came over between 117–122°C.

Distillation 2.

The residues boiling above 130°C., *i.e.* Fraction (e) from Distillation 1, were combined and fractionally distilled under reduced pressure. The whole of this batch, with the exception of a small residue, came over while the thermometer fluctuated between 77–79°C. The corrected boiling point of the fraction was found to be 163.3°C.

Esterification.—The combined fractions for the temperature interval 105–130°C. amounted to 22 cc. when recovered from the containers. An equal volume of ethyl alcohol and 1 cc. of concentrated H_2SO_4 were added to the fraction, and the mixture was digested for 3 hours in a small flask below a spiral condenser. After cooling under the tap the products of the digestion were neutralized with successive washings of dilute sodium carbonate. The ester layer was separated and shaken with a small volume of distilled water containing an excess of calcium chloride. The

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ester layer was again separated and placed over anhydrous calcium chloride for 24 hours. The upper layer, free from water and traces of alcohol, was then distilled at atmospheric pressure.

Distillation 3.

Volume of ester layer distilled.....	21.60 cc.
Fraction 74-80°C.....	14.30 "
Residue.....	4.40 "
Summary of results from Experiment 4.	
Temperature interval of first large fraction.....	117-122°C.
(B.P. of acetic acid).....	118 "
Temperature interval covered during the distillation of the ethyl ester prepared from this fraction.....	74-80 "
(B.P. of ethyl acetate).....	77 "
Corrected B.P. of the fraction obtained from D2.....	163.3 "
(B.P. of <i>n</i> butyric acid).....	163.0 "

These results indicate that during the fermentation acetic acid and a larger quantity of *n* butyric acid are formed. There remains however the possibility that smaller amounts of one or more other acids are formed. Although the experiments described were conducted with care, and as far as possible loss of material was avoided during the various stages, it is not considered advisable to draw any quantitative conclusions from the results obtained. They confirm the results of investigations by Henley (4) and his collaborators, who have also obtained results of a quantitative character. Our major problem has now been simplified and can be stated in the following terms. What is the relation, in the biochemical system of the fermentation, between acetic and *n* butyric acids and the neutral products of the fermentation, acetone and the two alcohols, ethyl and butyl?

Relation between the Formation of the Neutral Products and Changes in the Acidity of the Medium.

The following experiments were performed to find out the relation in time between the changing acidity of the mash and the rate of the formation of the neutral products.

Experiment 5.—25 pounds of meal were added to approximately 40 gallons of cold water, in a large copper fermentation vessel. The mixture was well stirred by means of a motor-driven apparatus, and the vessel contents were slowly raised to boiling point by means of live steam. The mash so

prepared was then sterilized in the same closed vessel for 3 hours at 7 to 8 pounds steam pressure. At the close of this period the volume of the batch was approximately 66 gallons, and after cooling the mash it contained roughly 4 per cent of solids. The culture added was 6 liters of an active culture of the bacillus in 5 per cent maize mash. During the fermentation the temperature of the vessel was maintained at 98°F. After 10 hours of the fermentation period the acidity of medium was determined at regular intervals. Samples, 150 cc. in volume, were withdrawn at intervals of 2 hours and distilled. The distillate collected from each of these was 50 cc. in volume and was made up accurately to 100 cc. with distilled water. The acetone content of each sample was determined by titration, using the Messinger method. The results obtained from this experiment are presented in Table V.

TABLE V.

Date.	Time after inoculation.	0.1 N NaOH required for 10 cc. sample.	Acetone in 150 cc. sample.
	<i>hrs.</i>	<i>cc.</i>	<i>gm.</i>
<i>1919</i>			
Apr. 9, 10.00 a.m.	10	1.15	0.005
12.00 n.	12	1.80	0.004
2.00 p.m.	14	2.65	0.005
4.00 "	16	3.71	0.008
6.00 "	18	4.36	0.010
8.00 "	20	5.06	0.021
10.00 "	22	5.59	0.049
11.30 "	23.5	5.60	
May 9, 9.00 a.m.	33	3.82	
10.00 "	34	3.74	0.256
12.00 n.	36	3.62	0.274
2.00 p.m.	38	3.19	0.317
4.00 "	40	3.15	0.309
6.00 "	42	2.90	0.326
8.00 "	44	2.85	0.331
June 9, 10.00 a.m.	58	3.13	0.356
12.00 n.	60		0.368

Experiment 6.—The last experiment was repeated with the exception that an effort was made to obtain results showing the rate of the formation of alcohols. The same tests were made as in Experiment 5 and in addition a number of larger samples, 2,000 cc. in volume, were distilled at regular intervals of time during the fermentation. About 300 cc. of distillate were collected from each one and redistilled from a 500 cc. flask, using as a still-head a three-section Young's fractionating column. The distillate from each sample was completely saturated with K_2CO_3 and allowed to stand over an excess for 60 hours in the refrigerator. The oil layer from each sample was separated and measured by volume. By the method

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previously described the weight of acetone, and therefore the volume, present in the 2,000 cc. samples was estimated. From these two figures it is possible to obtain, by difference, the volume of the mixture of the two alcohols in the total volume of neutral products in the sample. Owing to the presence of water in the oil the results so obtained do not represent dry, pure alcohol. The amount of water is not considerable and the results, in spite of this error, do give us the information required; *i.e.*, the relation between the rate of formation of the alcohols and the changes in acidity of the medium. The results of this experiment are given in Table VI.

TABLE VI.

Date.	Time after inoculation.	0.1 N NaOH required for 10 cc. sample.	Acetone per 150 cc.	Acetone per 2,000 cc.	Total oil per 2,000 cc.	Total alcohol per 3,000 cc.
	hrs.	cc.	gm.	cc.	cc.	cc.
<i>1919</i>						
Sept. 16, 9.30 a.m. . .	11	2.60	0.010	0.30	0.60	0.30
11.30 " . .	13	3.95	0.024	0.40	0.60	0.20
1.30 p.m. . .	15	4.80	0.040	0.66	1.00	0.34
3.30 " . .	17	5.39	0.068	1.00	2.50	1.50
5.30 " . .	19	5.33	0.111	1.85	3.60	1.75
7.30 " . .	21	4.90	0.174	2.90	7.60	3.70
9.30 " . .	23	4.25	0.243	4.05	10.80	6.75
10.30 " . .	24	3.94				
Sept. 17, 9.30 a.m. . .	35	1.85	0.616	10.27	31.00	20.73
11.30 " . .	37	2.06	0.628	10.47	33.50	23.03
1.30 p.m. . .	39	2.07	0.656	10.94	Lost.	
3.30 " . .	41	2.09	0.655	10.92	35.00	24.08
5.30 " . .	43	2.10	0.675	11.25	35.00	23.75
7.30 " . .	45	2.20	0.663*	11.05	35.00	23.95
Sept. 18, 9.30 a.m. . .	59	2.31	0.673	11.22	35.50	22.28
1.30 p.m. . .	63	2.35	0.652*	10.87	Lost.	

* Acetone low on account of loss during distillation. The results obtained in Experiment 6 are represented in the form of curves in Chart 4.

The curves in Chart 4 show that during the first phase of the fermentation period, when the hydrolysis of starch and the increase in the acidity of the medium are in progress, there is little formation of the alcohols or acetone. Towards the close of this phase formation commences at a slow rate. During the second phase, when we find a diminution in the amount of free acid, the neutral compounds are produced with great rapidity. This is also the time during which we have already observed a marked increase in the rate of gas production. It is interesting also to

compare the relative amounts of acetone and the alcohols in the early samples analyzed with the amounts in the final sample. We find that the early ones contain more acetone than alcohol; whereas the final sample contains approximately twice as much alcohol as acetone. These facts suggest that the formation of

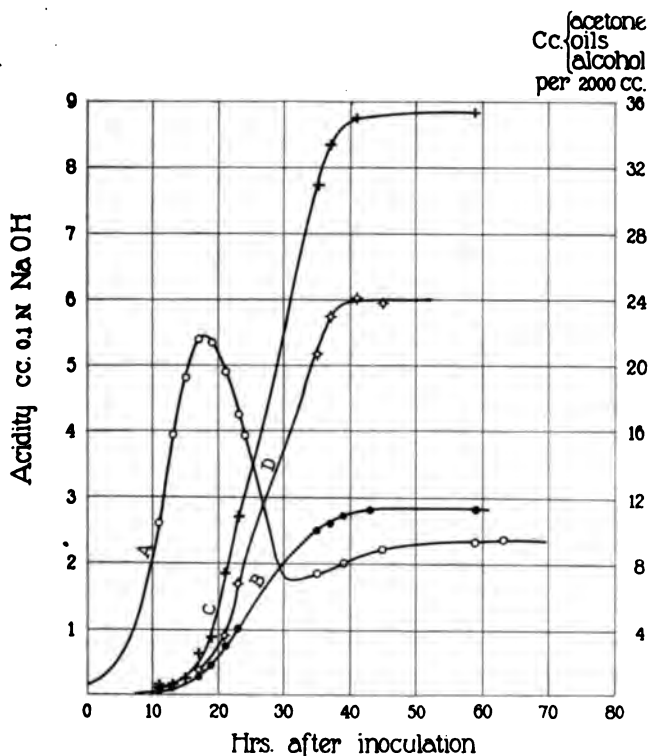


CHART 4. Curve A is the acidity curve and Curves B, C, and D the rate of production curves of acetone, total neutral products (oils), and alcohols respectively of the fermentation described in Experiment 6.

acetone commences slightly earlier than that of the alcohols. During the final phase of the fermentation, when the acidity of the medium rises very slowly to a constant, there is a very pronounced fall in the rate of production of acetone and alcohol until the end of the fermentation.

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Acidity Changes during the Fermentation of Mash Previously Acidified with Various Organic Acids.

A large number of experiments have been performed in which small volumes of organic acid have been added to the medium at different points in the fermentation period. For example, varying amounts of butyric acid were added to a number of flask cultures at a point when the acidity of the medium was falling rapidly. In a typical case an increase in acidity equivalent to 1.1 cc. of 0.1 N NaOH per 10 cc. of mash was brought about. This addition had no harmful effect on the culture, and, what is equally significant, the final acidity of the flask was normal, 10 cc. = 2.3 cc. of 0.1 N NaOH.

TABLE VII.

Date.	Time after inoculation.*	Cc. 0.1 N NaOH required for 10 cc. sample.		
		Control flask.	Acetic flask.	Butyric flask.
	hrs.	cc.	cc.	cc.
<i>1918</i>				
Jan. 16, 5.00 p.m.	0		3.60	3.50
" 17, 10.30 a.m.	17.5	3.60	4.00	3.40
12.00 n.	19	4.00	4.40	3.90
4.00 p.m.	23	3.60	5.00	4.30
Jan. 18, 10.00 a.m.	41	2.00	2.30	3.00
12.00 n.	43	2.20	2.20	2.30

* The flasks were inoculated at 5.00 p.m., Jan. 16, 1918.

In a second group of experiments the acid was added before inoculation. The acidity changes of each flask were followed, and as the experiment was repeated the initial acidity of the medium, due to free organic acid, was increased. Finally sufficient acid had been added to inhibit the life processes of the bacillus. In other flasks with a lower initial acidity, the fermentation was only retarded. The following is a description in greater detail of such a series of flasks.

Experiment 7.—Three flasks each containing 750 cc. of 5 per cent maize mash were sterilized for 2.5 hours at 10 pounds steam pressure. Flask A acted as a control, Flask B was acidified with acetic acid, and Flask C was acidified with butyric acid. The three flasks were inoculated at the same time with equal amounts of culture and were incubated together. The acidity of the flasks was determined at intervals. At the close of the

fermentation the flasks were similar in external appearance. The starch was completely hydrolyzed and the flasks contained clear yellowish green fluid upon which the slimy albuminous residue was floating. The results from this experiment are presented in Table VII.

The results show that during the fermentation of Flasks A and B a portion at least of the organic acid added to the flasks was involved in some biochemical reaction with the result that it was converted into some essentially different substance. Similar results were obtained when a sufficient amount of propionic acid was added to the mash previous to inoculation. An effort was then made to isolate and identify the compounds produced by the reaction involving the organic acids.

Quantitative Experiments Showing the Compounds Produced during the Fermentation of Mash Plus Various Organic Acids.

The object of the experiment was to determine the nature and volume of the neutral products of fermentations of maize mash to which known quantities of acetic, propionic, or butyric acid have been added previous to inoculation. The general method adopted was to ferment flasks of medium of known volume and concentration, to which the acid was added, and at the close of the fermentation to isolate, measure, and identify the various compounds by careful fractional distillation. The flasks were arranged in groups according to the nature of the organic acid added to the mash.

Experiment 8.—Group A.—10 liters of mash were prepared containing 400 gm. of maize meal. The mash was sterilized for 2.5 hours in the autoclave at 10 pounds steam pressure. This group was the control for the experiment.

Group B.—Eight flasks containing an equal volume of mash of the same concentration, and sterilized in the same manner. To each flask 2.5 cc. of glacial acetic acid were added previous to inoculation.

Group C.—Eight flasks similar to those in Group A, with 3.0 cc. of propionic acid added to each flask before inoculation.

Group D.—Eight flasks similar to the above, to which 3.0 cc. of butyric acid were added.

The acid was added when the flasks had cooled, and they were then inoculated, each with 50 cc. of an active culture of the bacillus in 5 per cent mash, used 20 hours after inoculation. The flasks of the four groups were incubated at 36.5°C., and during the fermentation period they were

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not disturbed in any way. In all cases the mash was completely fermented. The contents of the different groups of flasks were then examined in the following manner.

Distillation 1 (D1).—The 10 liters of beer from each group were distilled from a large copper vessel. The distillate was condensed in a straight glass tube condenser, and was collected until it no longer contained traces of butyl alcohol. Approximately 1,500 cc. were obtained from each group.

Distillation 2 (D2).—The distillate from D1 of each group was partially saturated with NaCl and redistilled from a flask. A three-section Young's fractionating column was used for a still-head. The distillation was continued in each case until all the butyl had passed over.

Distillation 3 (D3).—The second distillation was repeated using a smaller flask. The fractions boiling above and below 75°C. were collected in different receivers. The higher boiling fraction was shaken with a con-

TABLE VIII.

Temperature interval.	Volume of fraction.			
	Control.	Acetic group.	Propionic group.	Butyric group.
°C.	cc.	cc.	cc.	cc.
Below 65.	24.00	33.75	32.50	29.50
65-75	6.00	9.75	7.00	3.50
75-85	6.00	5.00	9.75	10.00
85-90.5	11.00	9.50	13.00	8.00
90.5-105	4.00	6.25	4.75	5.00
105-115	8.00	8.00	23.00	12.50
Above 115.	58.00	59.00	49.00	72.50
Total volume.....	117.00	131.25	139.00	141.00
Volume of acid added to mash.	0.00	20.00	24.00	24.00

siderable amount of K_2CO_3 and allowed to stand over an excess for 24 hours. The upper layer was separated from the aqueous layer and restored to the lower boiling fraction. The combined fractions were redistilled.

Distillation 4 (D4).—For this distillation a smaller column, made up of three bulbs each containing one loose, blown glass bead, was used. The temperature intervals adopted and the volumes of the fractions obtained for the four groups of flasks are embodied in Table VIII.

The small residue is in each case included in the final fraction. From the relatively small volume of the fraction obtained between 90.5-105°C. in each case we may conclude that the amount of water present was very small, and from the uniformity of the fractions from the four series that the amount still present in the

distillates was approximately the same in all cases. In the three series of flasks to which the acids were added previous to inoculation there was a considerable increase in the volume of neutral products of the fermentation. When the results of the experiment are considered in greater detail we find that (a) in the acetic series there was an increase of 45 per cent in the products boiling below 75°C., and smaller differences in the higher fractions when compared with those obtained from the control; (b) in the propionic series there was an increase of 31.7 per cent in the fractions boiling below 75°C., an increase of 187.5 per cent in the fraction boiling between 105–115°C., and a decrease in the fraction boiling above 115°C. of 15.5 per cent; and (c) in the butyric series there was an increase of 10 per cent in the fractions boiling below 75°C., and an increase of 25 per cent in the fraction boiling above 115°C. The results obtained from this experiment were verified three times before proceeding further with the research. An effort was then made to elucidate the nature of the compounds produced from the propionic acid series.

From the results obtained in the butyric acid series it was clear that there was a large increase in the production of butyl alcohol. There seemed to be strong evidence for the assumption that this was produced during the fermentation from the acid added to the mash. If propyl alcohol were produced from the acid added to the third series, in what fractions would it appear in the final distillation? In this connection it was recalled that of the ethyl alcohol produced during the normal fermentation the majority is found with butyl alcohol and water in the 85–91°C. fraction. Using pure propyl and butyl alcohols it was then found what results were obtained when a mixture of the two was fractionally distilled.

Experiment 9.—(A). A known volume of butyl alcohol was distilled using the apparatus which had been used in D3 and D4 of Experiment 8. The following figures relate to this distillation.

	cc.	cc.
Volume of butyl alcohol distilled.....	75.00	60.00
Fraction boiling below 105°C.....	0.00	0.00
“ “ from 105–115°C.....	3.00	4.50
“ “ above 115°C.....	72.50	55.50

(B). A mixture of the pure alcohols, with butyl alcohol in excess, was then distilled.

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Volume of butyl in the mixture.....	cc. 75.00	cc. 60.00
“ “ propyl “ “	5.00	5.00
Fraction boiling below 105°C.....	1.00	0.00
“ “ from 105–115°C.....	15.00	17.00

From these results it was concluded that when such a mixture of the two alcohols is distilled a large fraction containing both alcohols comes over between 105–115°C.

Experiment 10.—The object of this experiment was to obtain a larger volume of the unknown fraction in the products from the mash plus propionic acid fermentation. Mash was prepared containing 2,000 gm. of meal and was then made up to 50 liters. After sterilization the mash was allowed to cool and 120 cc. of propionic acid were added. The same per-

TABLE IX.

Temperature interval.	Volume of fraction.		Temperature interval.
	Experiment 10.*	Group 3, Experiment 8.	
°C.	cc.	cc.	°C.
57–65	197.00	32.50	Below 65.
65–75	12.00	7.00	65–75
75–85	35.00	9.75	75–85
85–95	26.00	13.00	85–90.5
95–105	47.00	4.75	90.5–105
105–115	43.50	23.00	105–115
Above 115.	297.00	49.00	Above 115.
Total volume..	657.50	139.00	

* The amounts used in Experiment 10 were five times those used in Group 3, Experiment 8.

centage of inoculant was added as in the last experiment and the fermentation was allowed to proceed undisturbed in the incubator at 36.5°C. The products of the fermentation were analyzed by distillation in the manner previously described. Traces of water were removed by salting out the alcohol from the 90.5–95°C. fraction. The results obtained from this experiment after the middle fractions had been redistilled several times are given in Table IX. The results obtained from D4, Experiment 8, of flasks containing propionic acid are included for comparative purposes.

The figures indicate that in the redistillation of the middle fractions, as the acetone, butyl alcohol, and water contained in them are eliminated, the relative volumes of these fractions change and progressive increases are observed in the 75–85 and 95–105°C.

fractions. As we have already observed, the accumulation of distillate in the first of these fractions is due to the gradual purification of the ethyl alcohol. The formation of the second of these large fractions was due to the presence of propyl alcohol which was gradually separated from the larger volume of butyl alcohol. From the fractions boiling above 95°C. shown in Table IX, 37.00 cc. of propyl alcohol were obtained.

Discussion of Experiments 8, 9, and 10.

In our consideration of the facts established by a comparison of the nature and volume of the compounds produced during the normal and abnormal fermentations, attention is called only to

TABLE X.

		per cent	per cent
Mash plus 0.2 per cent acetic acid by volume.	Increase in the yield of acetone.....	45.0	
	“ “ volume of ethyl alcohol in terms of acetic acid added.....		0.00
Mash plus 0.24 per cent propionic acid by volume.	Increase in the yield of acetone.....	30.0	
	“ “ “ “ propyl alcohol in terms of propionic added.....		30.0
Mash plus 0.24 per cent butyric acid by volume.	Increase in the yield of acetone.....	10.0	
	“ “ “ “ butyl alcohol in terms of butyric added.....		80.0

differences regarding the existence of which there does not appear to be room for doubt. With regard to the results obtained from the flasks containing mash plus acetic acid, a slight increase in the yield of butyl alcohol was observed, but further investigation is deemed necessary before this can be regarded as a characteristic result of such an experiment. There remains also the possibility that, working with larger volumes of mash to which a larger percentage of acetic acid has been added before inoculation, an increase in the production of ethyl alcohol might be produced.

Neglecting these possibilities, from the results obtained it has been calculated what the increase in the yield of acetone was in each group of flasks, and approximately what was the ratio between the alcohol equivalent of the amount of the organic acid

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added, and the increase in the production of the corresponding alcohol. It was assumed from the results obtained in Experiment 10 that, when 120 cc. of propionic acid are added to 50 liters of mash, 37 cc. of propyl alcohol are formed. This estimate disregards the propyl present in the final 99–115°C. fraction which was too small in volume to refractionate. The figures in Table X regarding the acetic and butyric experiments are based on the results of Experiment 8.

DISCUSSION.

From the foregoing experiments it is clear that the organism when growing in a medium rich in starch secretes an enzyme, or group of enzymes, which hydrolyzes the starch to glucose. The sugar passes into the cell and by its oxidation acetic and *n* butyric acids are produced.

It is interesting to compare the sugar and the two acids produced with the acids formed during the fermentation of xylose (5). In both cases the sum of the carbon atoms in the two acids is the number of carbon atoms in the sugar from which they are derived.

The acidity of the fermentation gradually rises. At a slightly later stage another process commences; namely, the conversion of the acids into substances which are neutral. Eventually the second process is more active than the first and there is for several hours a flow of acid into the cell from the medium. Towards the end of the fermentation the balance is again reversed and the acidity of the medium rises slightly once more. The method adopted for the determination of the acidity only serves to indicate the balance between the acid-forming and acid-destroying systems and does not furnish data regarding the true acidity of the normal fermentation.

The experiments showing the variations in the acidity of fermenting media of different concentrations, and of abnormal fermentations due to the temperature conditions or the low vitality of the organism, indicate that the formation and destruction of the acids are not independent of but are essential parts of the biochemical system responsible for the formation of the alcohols and acetone. It has been shown that the period during which the destruction of the acids is most active is the period during

which the neutral products are being most rapidly formed. These facts can be explained by the hypothesis that the organic acids are intermediate compounds in the fermentation.

We shall discuss in the first place the evidence supporting the hypothesis that the acids are reduced to the alcohols. When acetic, propionic, and butyric acids were added to different portions of mash, equal in volume and concentration, it was found that there was no change in the yield of ethyl alcohol, that propyl alcohol was formed, and that there was a large increase in the yield of butyl alcohol. Now if the acids give rise to the alcohols in the normal fermentation, why was there no marked increase in the yield of ethyl alcohol when acetic acid was added to the mash? In order to increase the yield of the corresponding alcohol the acid must pass through the cell wall. What are the factors present under the conditions obtaining, which are known to influence the penetration of cell walls by acid? The cells were immersed in solutions of acids, present in varying concentrations. When the flow of acid into the cells started in the acetic flasks the order of concentration was: butyric = acetic; in the propionic flasks: butyric = propionic > acetic; and in the butyric flasks: butyric > acetic. Crozier (6) has shown, working with animal cells and 0.01 N solutions of the fatty acids, that the order of penetration among the lower members of the series is as follows: valeric > butyric > propionic > acetic. Harvey (7) has obtained similar results, also working with animal cells. Haas (8) arrived at a similar conclusion from experiments on the penetration of living plant cells by 0.01 N solutions, made by titration, of acids and alkalies. Very different results were obtained when the acid solutions were standardized by the gas chain method. If the same law of penetration applies when bacteria are used, and if the acid passing through the wall is converted to the corresponding alcohol, then the increases in the yields of the alcohols, expressed as ratios of the alcohol equivalents of the amounts of the different acids added to the mash, should correspond to the order of penetration. The figures in the right hand column of Table X do so in a very striking manner. Reilly (4) has observed that in the later part of the fermentation, *i.e.* when acid is passing into the cell, the percentage of acetic acid in the total free acid increases. The results of these experiments show that during the normal fermentation the two acids are reduced to the corresponding alcohols.

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We shall now consider the results in relation to the production of acetone. We observe that during the rapid destruction of the acids formed during the normal fermentation there is a corresponding production of acetone, and that without the first the second does not occur. Is it possible that acetone is also formed from one or both of the acids? From the flasks to which various acids were added largely increased yields of acetone were obtained. Reilly (4) has made similar observations in connection with acetic acid. He states:

"It appeared that the bacteria or enzymes were actually able to reduce the acetic acidity in considerable quantities. The percentage of acetone was increased by the addition of acetic acid. If acetic acid were made by an ordinary fermentation process, for example, by souring cheap wines, the dilute solution could be pumped directly into the fermentation vat for conversion into acetone."

Our results show that if we assume a conversion of acetic acid into acetone, we must in the end assume the presence in the cell of some system by which acetone is formed from the three acids, acetic, propionic, and butyric. This is highly improbable. Furthermore if the acid passing through the cell wall is partially converted into acetone we should expect the largest increase in yield from the butyric acid flasks, for, as we have observed earlier in the discussion, the acids penetrate the cell wall in increasing amounts as we ascend the series. It was found that the increase in the yield of acetone diminishes in a regular manner as we ascend the series. For these reasons I conclude that the yield of acetone is affected by influences exerted by an acid on intercellular life by virtue of its properties and presence in the surrounding solution only and not by conversion into acetone within the cell.

Although the acetone is not produced from acid which passes from the medium into the cell, is it formed from organic acid which flows directly from the acid-producing to the acetone-producing region in the cell. It is also necessary to assume that the acid passing into the cell does not reach the acetone system. Such an organization might be influenced in the manner described by the presence of large quantities of acid in the mash before inoculation, for example, by restricting the flow of acid from the cell. For theoretical reasons it does not seem probable that acetone is produced from acetic acid, but there remains the possibility with

regard to butyric acid. Witzemann (9) has shown that acetone is produced by the oxidation of butyric acid by means of hydrogen peroxide. It would seem advisable however to leave this portion of our problem for further investigation.

CONCLUSIONS.

From the results obtained by this investigation we conclude that:

1. *Bacillus granulobacter pectinovorum* growing in a medium rich in starch changes the latter into glucose by exoenzyme activity.
2. Glucose passes into the cell and is oxidized to acetic and butyric acids.
3. These acids are in part reduced to the corresponding alcohols.

I wish to thank my colleague Mr. A. M. Wynne for his assistance during the prosecution of this research.

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DETERMINATION OF CHLORIDES IN WHOLE BLOOD.

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(Received for publication, January 26, 1920.)

As it is desirable at times to determine the chloride content of the whole blood rather than of the plasma only, the applicability to this purpose of the Van Slyke-Donleavy method¹ for the determination of plasma chlorides was investigated.

It was found that some component of the laked cells other than the chloride has the property of binding silver, so that the direct application of the Van Slyke-Donleavy method to whole blood gives readings too high by 30 to 40 per cent. If, however, after laking, the protein is precipitated by picric acid alone, or by picric and nitric acids, and the protein-free filtrate is treated with silver nitrate, a quantitative precipitation of the total chloride of the whole blood is obtained and the final titration may be carried out as in the Van Slyke-Donleavy method. The accuracy of this modification was tested on whole oxalated ox blood by comparison with chloride estimation by the Carius method, using the technique for destroying the organic matter adopted by Vinograd² and a Volhard titration of the silver remaining unbound.

Carius Method.

Whole oxalated ox blood was analyzed for chloride content by the Carius method as follows.

Approximately 1 cc. of the whole blood was introduced from a pipette into the bottom of a bomb tube and the amount accurately determined by weighing the pipette before and after delivery.

¹ Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

² Vinograd, M., *J. Am. Chem. Soc.*, 1914, xxxvi, 1548, also *Studies from The Rockefeller Institute for Medical Research*, 1915, xxii, 372.

1 cc. of a solution of silver nitrate containing 20.4 mg. of AgNO_3 per cc. was carefully introduced into the bottom of the tube. The tube was then immersed in a water bath at 100° and the moisture-laden air in the upper portion of the tube continually removed by aspiration until the water was driven from the mixture of blood and silver nitrate. 1 cc. of fuming nitric acid in a small tube was then introduced into the tube. The tube after being sealed was slowly heated to 180° and this temperature maintained for 3 hours. After cooling, opening, and washing out the colorless solution with distilled water the remaining unprecipitated silver was titrated by Volhard's method with a 0.015 M solution of NH_4CNS , the chloride obtained by difference being expressed as NaCl . The results of four determinations are given in Table I.

Results with Modified Van Slyke-Donleavy Method.

Into each of two 50 cc. volumetric flasks were introduced 4 cc. of the same whole oxalated ox blood used in the Carius analysis and the weight of blood introduced was determined by weighing the flasks before and after introduction of the blood. After laking by addition of 20 cc. of distilled water, there were added 20 cc. of the following solution of nitric and picric acids:

	cc.
HNO_3 (sp. gr. 1.42).....	250.0
Picric acid.....	7.5
Distilled water to.....	1,000.0

The flask was then filled to the mark with distilled water and repeatedly inverted. After allowing 10 minutes for complete precipitation of the protein, the mixture was filtered through a dry filter and 25 cc. of the clear yellow filtrate were introduced into a 50 cc. volumetric flask. To this were added 10 cc. of an $\text{M}/29.25$ AgNO_3 solution, the flask was filled to the mark, and two drops of caprylic alcohol were added. After standing over night (to promote clear filtration) the clear supernatant fluid was removed with a pipette, filtered, and two 20 cc. portions were taken. These were titrated with KI solution after addition of 4 cc. of the special starch solution as described in the Van Slyke-Donleavy method.

The results of four determinations are given, together with those on the same blood by the Carius method, in Table I. In the last column are given also the results obtained in attempting to apply the Van Slyke-Donleavy method, without modification, to whole blood.

The chief difficulty encountered in the present method was in securing a clear filtrate after the precipitation of the AgCl. This is probably somewhat facilitated by using more picric acid and

TABLE I.

Results of Chloride Determinations on a Specimen of Whole Blood.

Carius method.			Van Slyke-Donleavy method modified for whole blood by precipitating proteins and AgCl separately.		VanSlyke-Don- leavy method for plasma ap- plied to whole blood without modification.
Weight of blood.	NaCl found.	NaCl found per gm. of blood.	Weight of blood.	NaCl found per gm. of blood.	NaCl found per gm. of blood.
gm.	mg.	mg.	gm.	mg.	mg.
0.998	4.47	4.48	4.108	{ 4.53	6.00
1.000	4.54	4.54		{ 4.53	5.95
1.005	4.57	4.55	4.113	{ 4.54	5.98
1.002	4.57	4.56		{ 4.53	6.05
Average		4.53		4.53	5.99

less nitric acid for the precipitation of the protein, then adding the required amount of nitric acid to the final 20 cc. portions just before addition of the starch solution. The most effective means of securing a clear filtrate, however, is by permitting the solution to stand in the dark over night after the addition of the silver nitrate; a perfectly clear supernatant liquid can then be pipetted from the flask.

The technique finally adopted is as follows.

Method for Determination of Chlorides in Whole Blood.

Take 3 cc. of blood with 15 cc. of water in a 60 cc. flask. Add 30 cc. of saturated picric acid solution and sufficient water to bring the volume to 60 cc. (27 cc. of water in all, and 30 cc. of picric acid may be measured from burettes if a 60 cc. measuring flask is not available). The contents of the flask are mixed, and after 10 minutes are filtered.

To 40 cc. of the filtrate 10 cc. of the $M/29.25$ silver nitrate solution used by McLean and Van Slyke³ are added, with two drops of caprylic alcohol (the silver solution contains per liter 5.812 gm. of $AgNO_3$ and 250 cc. of HNO_3 (sp. gr. 1.42). The solutions are thoroughly mixed, and preferably allowed to stand over night to allow the $AgCl$ to coagulate and settle. The supernatant solution is decanted through a small filter paper, and 20 cc. are titrated as described by Van Slyke and Donleavy. The calculation also is the same, since the 20 cc. of filtrate titrated in this case, as in the method of Van Slyke and Donleavy, represent 0.8 cc. of the original material, blood or blood plasma.

Attention may be called to the fact that the use of the unmodified Van Slyke-Donleavy method with a plasma that is considerably stained as the result of partial hemolysis of the cells will give values that are unduly high.

CONCLUSIONS.

1. The method described by Van Slyke and Donleavy for the determination of plasma chlorides is not directly applicable to whole or to laked blood.

2. A modified technique for whole or laked blood is described in which the precipitation and removal of the protein precede the addition of the silver.

³ McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

EXPERIMENTS ON THE UTILIZATION OF THE CALCIUM OF CARROTS BY MAN.

BY MARY SWARTZ ROSE.

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(Received for publication, January 24, 1920.)

McClugage and Mendel¹ have recently called attention to the scantiness of our knowledge concerning the value of the calcium of vegetables for animal nutrition, and have described experiments with dogs in which the calcium was very poorly utilized when derived from carrots.

When that paper was published two of the four experiments reported herein had been completed, but as the results were strikingly different the work was continued with two other subjects. Each of the four subjects had shortly previous to the calcium experiment been the subject of digestion experiments with simple mixed diets, and their digestive capacity was known to be high. The carrot was chosen for special study because it is ingested comparatively easily in large amounts over considerable periods, and because it is a vegetable with a high calcium content.

Methods.

The general plan was to arrange an easily digested diet with a calcium content approximately equal to the estimated minimal requirement for equilibrium in each subject, and to determine calcium balances from the calcium content of analyzed food, urine, and feces. In Series I with two subjects, there were two periods of 3 weeks each. In Period I, milk was made the chief

¹ McClugage, H. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 353.

source of calcium; in Period II, the milk was largely omitted and carrots were introduced to make up the full quota of calcium. It was thought that if the subjects were about in equilibrium on the milk ration, any failure to utilize the calcium would show promptly in the calcium balance. In Series I, the milk period was omitted and the carrot diet consumed for 2 weeks.

Sherman has pointed out² that in 63 experiments on ten subjects calcium equilibrium could be maintained on amounts ranging from 0.27 to 0.78 gm. of calcium per 70 kilos of body weight per

TABLE I.
Calcium Content of Foods Used in Diets.

Food material.	Calcium.
	<i>per cent</i>
Bread.....	0.032
Beef, lean.....	0.018,* 0.009,† 0.004‡
Milk.....	0.116
Honey, strained.....	0.004
Butter.....	0.014,* 0.010†
Peaches.....	0.006
Apple.....	0.010,* 0.0024†
Tomato juice.....	0.015
Rice.....	0.012
Coffee infusion.....	0.003
Carrots.....	0.044,* 0.052†
Soda crackers.....	0.025

* Series I.

† " II.

‡ " II, Periods II and III.

day with an average of 0.45 gm. In these experiments the milk diets yielded 0.48 and 0.50 gm. per 70 kilos and the carrot diets from 0.39 to 0.46 gm. Only distilled water was used throughout.

Urine and feces were collected daily and combined into 4 day periods for analysis. The carrots were boiled in their skins, in just enough water to steam them, then the skins removed, the carrots mashed, mixed, and sampled for analysis. Calcium was determined by McCrudden's method.

The calcium content of the foods used in the daily diets is given in Table I.

² Sherman, H. C., *Chemistry of food and nutrition*, New York, 2nd edition, 1918, 264.

EXPERIMENTAL.

In Series I, the two young women had so nearly the same energy requirement that they decided to eat exactly the same amounts of food throughout. The time was divided into two periods of 3 weeks each. In Period I, 70 per cent of the calcium intake came from milk; in Period II, 21 per cent from milk and 55 per cent from carrots. The calcium content of the daily diets is given in Table II.

TABLE II.
Calcium Content of Daily Diets. Series I.
Subjects E. D. B. and R. S. E.

Food.	Period I.		Period II.	
	Weight.	Calcium.	Weight.	Calcium.
	gm.	gm.	gm.	gm.
Bread.....	152	0.0476	152	0.0477
Meat.....	100	0.0169	50	0.0086
Milk.....	226	0.2644	57	0.0668
Honey.....	31	0.0011	31	0.0015
Butter.....	50	0.0072	50	0.0072
Sugar.....	30		50	
Rice.....	57	0.0069	29	0.0035
Peaches.....	106	0.0068		
Apple.....	100	0.0100		
Tomato juice.....	100	0.0150		
Coffee infusion.....	200	0.0063	200	0.0057
Carrots.....			400	0.1744
Total per day		0.3822		0.3154
“ “ kg. R. S. E		0.0068		0.0056
“ “ “ E. D. B		0.0071		0.0058

The lower calcium intake in Period II was regrettable as it introduced the possibility of an actual calcium deficiency, but the final balances showed that requirement was fully met in the case of E. D. B., though perhaps not quite in that of R. S. E. This change came about through the inability of the subjects to consume as large amounts of the carrots as they had intended. On account of the bulkiness of the diet, the rice was reduced in Period II. The tomato juice, peaches, and apple used in Period I to keep the diet laxative were dispensed with when carrots were introduced.

Both subjects lost calcium during the first 4 day period. This was attributed to their changing from a high to a low calcium

TABLE III.
Daily Intake and Output of Calcium, Series I.
Subject E. D. B., weight 54 kg.

Series.	Period.	Time.	Average daily intake of calcium.	Average daily output of calcium.			Calcium balance.
				Urine.	Feces.	Total.	
		<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
I	I	4	0.383	0.086	0.553	0.639	-0.225
	II	4	0.383	0.081	0.290	0.371	+0.012
	III	4	0.383	0.071	0.243	0.313	+0.070
	IV	4	0.383	0.069	0.215	0.284	+0.099
	V	5	0.383	0.056	0.267	0.323	+0.060
Average.....	II-V	17	0.383	0.070	0.254	0.323	+0.060
II	VI	4	0.315	0.033	0.133	0.166	+0.149
	VII	4	0.315	0.045	0.165	0.210	+0.105
	VIII	4	0.315	0.076	0.236	0.312	+0.004
	IX	4	0.315	0.076	0.235	0.311	+0.003
	X	5	0.315	0.060	0.239	0.299	+0.016
Average.....	VI-X	21	0.315	0.058	0.202	0.260	+0.055

Subject R. S. E., weight 56 kg.

I	I	4	0.383	0.064	0.567	0.632	-0.249
	II	4	0.383	0.070	0.264	0.334	+0.049
	III	4	0.383	0.069	0.242	0.311	+0.072
	IV	6	0.383	0.069	0.191	0.260	+0.121
Average.....	II-IV	14	0.383	0.069	0.226	0.296	+0.087
II	V	4	0.315	0.074	0.287	0.361	-0.046
	VI	4	0.315	0.077	0.189	0.266	+0.049
	VII	4	0.315	0.073	0.327	0.400	-0.084
	VIII	4	0.315	0.072	0.220	0.292	+0.023
	IX	5	0.315	0.079	0.287	0.366	-0.050
Average.....	V-IX	21	0.315	0.075	0.262	0.336	-0.022

intake and attaining equilibrium on a new level, and the period has been excluded from the averages for the period, though its inclusion would make apparently a better case for the calcium

of carrots. No such loss occurred on going from the milk to the carrot diet. Some loss at this time occurred in the case of R. S. E. who was the most liable to digestive disturbances of all the subjects, and found the carrot diet slightly less satisfactory than the others. The data for the calcium balances are given in Table III.

TABLE IV.
Calcium Content of Daily Diets. Series II.
Subject E. H.

Food.	Period I.		Period II.		Period III.
	Weight.	Calcium.	Weight.	Calcium.	Calcium.
	gm.	gm.	gm.	gm.	gm.
Meat.....	100	0.0090	150	0.0100	0.0060
Crackers.....	85	0.0213	100	0.0250	0.0250
Butter.....	70	0.0070	100	0.0100	0.0100
Sugar.....	100		100		
Apple.....	100	0.0024			
Carrots.....	466	0.2423	400	0.2080	0.2080
Total per day		0.2820		0.2530	0.2490
“ “ kg.....		0.0063		0.0056	0.0055

Subject E. W.

Food.	Period I.		Periods II and III.	
	Weight.	Calcium.	Weight.	Calcium.
	gm.	gm.	gm.	gm.
Meat.....	100	0.0090	100	0.0040
Soda crackers.....	150	0.0375	150	0.0375
Sugar.....	100		100	
Apple.....	100	0.0024	100	0.0024
Carrots.....	400	0.2080	533	0.2770
Total per day		0.2569		0.3209
“ “ kg.....		0.0054		0.0067

Digestive disturbances at the beginning and end of Period II (with carrots) in the case of R. S. E. show their effect in the negative calcium balance in both periods. It is worth noting, however, that on this low calcium intake distinct gains were made in three of the five periods and that if all the loss is charged to the carrot calcium the amount retained still represents 88 per cent of the intake.

In Series II, the allowance of calcium was practically the same as in the carrot diets of Series I. For lack of time, the milk period had to be omitted and the two young women who served as subjects subsisted for 12 days on a diet in which carrots furnished from 78 to 86 per cent of the total calcium. The daily diets are given in Table IV.

Slight changes in the diet were made after the first 4 days as E. H. found she could not eat so much carrot and E. W. that she could eat more. E. H. found the carrot diet sufficiently laxative

TABLE V.
Daily Intake and Output of Calcium. Series II.

Period.	Time.	Average daily intake of calcium.	Average daily output of calcium.			Calcium balance.
			Urine.	Feces.	Total.	
Subject E. H., weight 45.5 kg.						
	<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
I	4	0.282	0.058	0.198	0.256	+0.026
II	4	0.253	0.075	0.125	0.200	+0.053
III	4	0.249	0.081	0.217	0.298	-0.049
Average I-III..	12	0.261	0.071	0.180	0.251	+0.010
Subject E. W., weight 48 kg.						
	4	0.257	0.083	0.166	0.249	+0.008
II	4	0.321	0.062	0.160	0.222	+0.099
III	4	0.321	0.050	0.130	0.180	+0.140
Average I-III..	12	0.300	0.065	0.152	0.217	+0.082

without the apple used for that purpose in Period I. The meat used was somewhat fatter and had a lower calcium content in the later periods. The daily intake and output for each subject are shown in Table V.

SUMMARY.

Two series of experiments to determine the utilization of the calcium of carrots by the human body have been carried out on four healthy young women. The calcium intake was in every case close to the estimated minimum for equilibrium.

Average Daily Intake and Output of Calcium.

Series.	Subject.	Diet.	Average daily intake of calcium.	Calcium.					
				From carrots.	Average daily output.			Balance.	Gain or loss.
					Urine.	Feces.	Total.		
			gm.	per cent	gm.	gm.	gm.	gm.	per cent
I	E. D. B.	Milk.	0.383		0.070	0.254	0.323	+0.060	+15.6
I	"	Carrot.	0.315	55	0.058	0.202	0.260	+0.055	+17.4
I	R. S. E.	Milk.	0.383		0.069	0.226	0.296	+0.087	+22.7
I	"	Carrot.	0.315	55	0.075	0.262	0.336	-0.023	- 7.3
II	E. H.	"	0.261	84	0.071	0.180	0.251	+0.010	+ 3.8
II	E. W.	"	0.300	86	0.065	0.152	0.217	+0.082	+26.6

In all cases but one there was a positive calcium balance on the carrot diet, and in this case the loss was small. When approximately 55 per cent of the calcium was derived from carrots, one subject had practically the same retention as on a diet in which 70 per cent of the calcium was derived from milk. It seems possible, therefore, to meet the requirement of the adult human organism for calcium largely, if not wholly, from carrots.



EFFECTS OF FEEDING WITH CALCIUM SALTS ON THE CALCIUM CONTENT OF THE BLOOD.

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The advent of micro methods for the determination of blood calcium has opened up many interesting lines of work in a field hitherto practically closed on account of the relatively large amount of blood required for a single analysis, when the older gravimetric procedures are used. Somewhat more than a year ago there was begun in this laboratory a systematic study of the calcium content of the blood of persons suffering from a number of maladies for the relief of which calcium administration has been advocated, and of the effect on the blood calcium produced by the administration of calcium salts to these patients.

As a necessary preliminary to the interpretation of the results obtained on pathological material, we have carried out a number of experiments along the same lines on normal individuals and on animals, the results of which are described in the present paper. Our calcium determinations have been made by Lyman's (1) method, which has been modified in a few minor details.

All determinations have been made on citrated plasma (0.1 gm. of solid sodium citrate to 10 cc. of blood) as preliminary experiments convinced us that no calcium was precipitated by the use of this amount of sodium citrate, and that, as pointed out by Halverson, Mohler, and Bergeim (2), the determination of calcium in plasma or serum is of more significance than when made on whole blood, as originally recommended by Lyman.

In Table I we have collected the results obtained on five men and one woman, all normal subjects, who took by mouth daily three 2 gm. portions of calcium lactate, for a period of 6 to 10 days. During the period of calcium ingestion these subjects were under no regulations as regards food, but continued to eat their ordinary mixed diet.

As will be seen no effect on blood calcium was obtained by the daily ingestion of 6 gm. of calcium lactate in our normal human subjects. Attempts to give larger doses to men proved unsuccessful on account of the disagreeable taste of the various calcium salts tried, so that we were obliged to turn to animals for further experiments on the effects of relatively high calcium dosage.

As experimental subjects we have used cats and rabbits, as representing types of animals that ordinarily eat food differing widely in calcium content. These animals were kept in the

TABLE I.

Experimental Results Obtained on Feeding Calcium Lactate (6 Gm. per Day) to Normal Subjects.

Subject No.	Time.	Calcium per 100 cc. of plasma.
	<i>days</i>	<i>mg.</i>
1	1	7.6
	6	7.6
2	1	9.2
	6	9.1
3	1	11.0
	6	10.8
4	1	8.6
	6	9.2
5	1	11.8
	6	11.9
6	1	11.7
	10	11.8

laboratory and fed on the diet to be used during the experiment for 1 week before the initial samples of blood were taken. The food of the rabbits consisted of oats, cabbage, celery tops, and lettuce. The cats were given cooked lean meat (beef and mutton) in unmeasured amounts and in addition each cat received 50 cc. of milk per day.

In Table II we have collected the data obtained on seven cats to which calcium lactate was fed for periods varying from 15 to 38 days. While the number of experimental animals is rather

limited it would seem that it is sometimes possible by feeding with calcium salts to increase the calcium content of the blood of these animals, but that in certain instances calcium administration, even when continued for long periods, is without effect. There is apparently some relation between the initial calcium

TABLE II.

Experimental Results Obtained on Feeding Calcium Lactate to Cats.

Cat No.	Date.	Calcium per 100 cc. of plasma.	Remarks.
	1919	mg.	
1	Mar. 2	4.1	Male, weight 3,200 gm.; 0.5 gm. calcium lactate per day.
	" 27	7.3	
	June 11	5.2	2nd experimental period; 1.0 gm. calcium lactate per day.
	" 29	14.2	
2	Apr. 7	6.1	Female, weight 1,820 gm.; 0.5 gm. calcium lactate per day.
	" 27	7.2	
	June 11	7.6	2nd experimental period; 1.0 gm. calcium lactate per day.
	" 29	10.6	
3	Apr. 27	4.8	Male, weight 6,300 gm.; 0.5 gm. calcium lactate per day.
	May 12	5.7	
4	June 19	10.3	Female, weight 1,750 gm.; 0.5 gm. calcium lactate per day.
	" 29	10.4	
	July 11	11.4	2nd experimental period; 1.0 gm. calcium lactate per day.
	" 24	11.8	
5	Sept. 26	10.8	Male, weight 3,000 gm.; 1.0 gm. calcium lactate per day.
	Dec. 2	10.9	
6	Sept. 26	10.4	Male, weight 2,320 gm.; 1 gm. calcium lactate per day.
	Dec. 2	10.3	
7	July 26	11.0	Female, weight 1,950 gm.; 1.0 gm. calcium lactate per day.
	Dec. 2	11.4	

content of the plasma and the ease with which it is possible to effect changes in the blood by experimental means. Thus it will be noted that the calcium content of the plasma of Cats 1, 2, and 3 was rather low in the first sample, and that these animals responded positively to the administration of calcium salts,

whereas the plasma calcium of Cats 5, 6, and 7, for the species of animal, is rather high in initial value, and that calcium feeding was with them without demonstrable effect on the blood.

TABLE III.

Experimental Results Obtained on Feeding Calcium Lactate to Rabbits.

Rabbit No.	Date.	Calcium per 100 cc. of plasma.	Remarks.
	1919	mg.	
1	Feb. 16	11.2	Male, weight 2,100 gm.; 0.5 gm. calcium lactate per day.
	" 28	11.0	
18	July 24	13.5	Male, weight 1,800 gm.; 1.0 gm. calcium lactate per day.
	" 29	15.7	
4	Apr. 9	8.3	Female, weight 1,800 gm.; 0.5 gm. calcium lactate per day.
	" 27	8.2	
5	June 2	14.4	Female, weight 1,920 gm.; 1.0 gm. calcium lactate per day.
	" 11	14.2	
17	Sept. 25	14.4	Male, weight 2,240 gm.; 1.0 gm. calcium lactate per day.
	Dec. 1	14.3	
19	Sept. 25	16.0	Male, weight 2,100 gm.; 1.0 gm. calcium lactate per day.
	Dec. 1	15.8	
20	Sept. 25	8.2	Female; 5 young born 1 week before beginning of experiment; lactation continued for 4 weeks.
	Dec. 1	16.0	
21	Sept. 25	14.8	Female, weight 1,660 gm.; 1.0 gm. calcium lactate per day.
	Dec. 1	16.0	
22	Sept. 26	9.1	Female; on Oct. 6th delivered 6 young, which she nursed for about 4 weeks.
	Dec. 2	11.6	

The experimental results obtained on nine rabbits are summarized in Table III. As in the case of the cats but little effect on plasma calcium can be seen except in the case of Rabbits 20 and 22, both of which were lactating during a considerable portion of the experimental period, a fact which may account for the low initial values.

SUMMARY.

The result of a study of the effect of the administration of calcium salts by mouth to men, cats, and rabbits indicates that in most cases it is impossible to increase the concentration of calcium in the plasma by ingestion of calcium salts, but that in cats and rabbits where the initial concentration is low it is sometimes possible to greatly increase the amount of calcium in plasma by feeding salts of this element.

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DETERMINATION OF MAGNESIUM IN BLOOD.

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(Received for publication, January 30, 1920.)

In the course of an investigation of certain problems of calcium metabolism it became desirable to make determinations of magnesium in small amounts of plasma. The method described below has been worked out with this end in view, and has been adapted for use with the filtrate obtained after the precipitation of calcium in plasma or whole blood by Lyman's (1) method.

Briefly stated the procedure consists of the removal of organic material contained in the filtrate from the calcium determination, the precipitation of magnesium as magnesium ammonium phosphate, and the nephelometric determination of the phosphate in this compound by the reagent of Pouget and Chouchak (2). The detailed description of the method follows.

5 cc. of citrated plasma, serum, or whole blood are measured into 15 cc. of 6.5 per cent trichloroacetic acid solution, and after shaking the mixture is allowed to stand for at least 30 minutes, and is then filtered through a dry filter. 10 cc. of this filtrate (equivalent to 2.5 cc. of serum) are used for the determination of calcium according to the method of Lyman. In this method calcium is precipitated essentially according to the well known technique of McCrudden (3) and the crystals of calcium oxalate are then collected and washed by the help of the centrifuge. For the determination of magnesium the supernatant liquid remaining in the centrifuge tubes is collected by means of a small siphon, as is also the portion of ammonium oxalate solution used as wash liquid; the total amount of fluid collected in this way is placed in a flat-bottomed platinum dish and evaporated to dryness after the addition of 3 cc. of 10 per cent sulfuric acid; the residue in the dish is then ignited over a free flame until white, an operation which should be complete in 2 or 3 minutes. When cool the

white residue is dissolved in about 5 cc. of distilled water, and 10 per cent hydrochloric acid is added drop by drop until the solution of ash is acid to methyl orange. This solution is transferred quantitatively to a 100 cc. beaker, using distilled water to conclude the operation, and evaporated to a volume of 2 to 3 cc.; concentrated ammonium hydroxide is then added drop by drop until the solution is alkaline, and finally 0.5 cc. of 10 per cent ammonium phosphate solution containing 50 cc. of concentrated ammonium hydroxide per liter. The beaker is covered with a watch-glass and allowed to stand over night. The next day the liquid is poured into a conical centrifuge tube, and the beaker washed with 20 per cent alcohol containing 50 cc. of concentrated ammonium hydroxide per liter. After centrifuging, the liquid in the tube is removed by means of a small siphon, and the beaker and tube are again washed with about 10 cc. of the alcohol-ammonia mixture. Three more portions of wash liquid should be used, the precipitate being thoroughly stirred after each addition of fresh liquid.

The precipitation and subsequent washing just described are essentially the process described by Marriott and Howland (4) in their micro method for the determination of magnesium in blood.

This portion of the procedure is at once the most important and the most unsatisfactory feature of the determination. Both in the method of Marriott and Howland and in the procedure described in this paper, recourse has of necessity been had to a principle which every chemist will recognize as theoretically incorrect; *viz.*, the determination of magnesium by the measurement of the phosphate combined as ammonium magnesium phosphate, the precipitation of which latter compound must be carried on in the presence of a large excess of phosphate. It is obvious that extremely careful work is essential in the washing of the precipitate in order to avoid contamination with residual traces of ammonium phosphate; furthermore as is well known, it is possible to overwash an ammonium magnesium phosphate precipitate, so that low results are obtained.

After the removal of the last portion of wash liquid, the tube and beaker are allowed to stand, preferably on a water bath or register until the ammonium has evaporated, and the ammonium magnesium phosphate is then dissolved in 10 cc. of 0.1 *N* hydrochloric acid and transferred to a 100 cc. volumetric flask by means of distilled water. The solution is then made to volume with

distilled water, mixed, and the phosphate determined by means of the strychnine molybdate reagent.¹

For the determination of magnesium in normal plasma or serum 25 cc. of the above solution are usually the most convenient to use, but larger or smaller amounts are sometimes called for.

To the amount of solution taken is added a quantity of distilled water sufficient to bring the volume to 50 cc., then 25 cc. of the strychnine molybdate reagent are added. After standing for a period of 5 minutes the suspension is read against a standard containing 0.01 mg. of magnesium in a volume of 50 cc.² to which have been added 25 cc. of the strychnine molybdate reagent, and which has been allowed to stand for the same length of time as the unknown.

The procedure as outlined above has been carried out in pure solutions containing 0.02 to 0.10 mg. of magnesium, and on similar amounts of magnesium added to serum and plasma. The average recovery in these experiments has been 94 per cent.

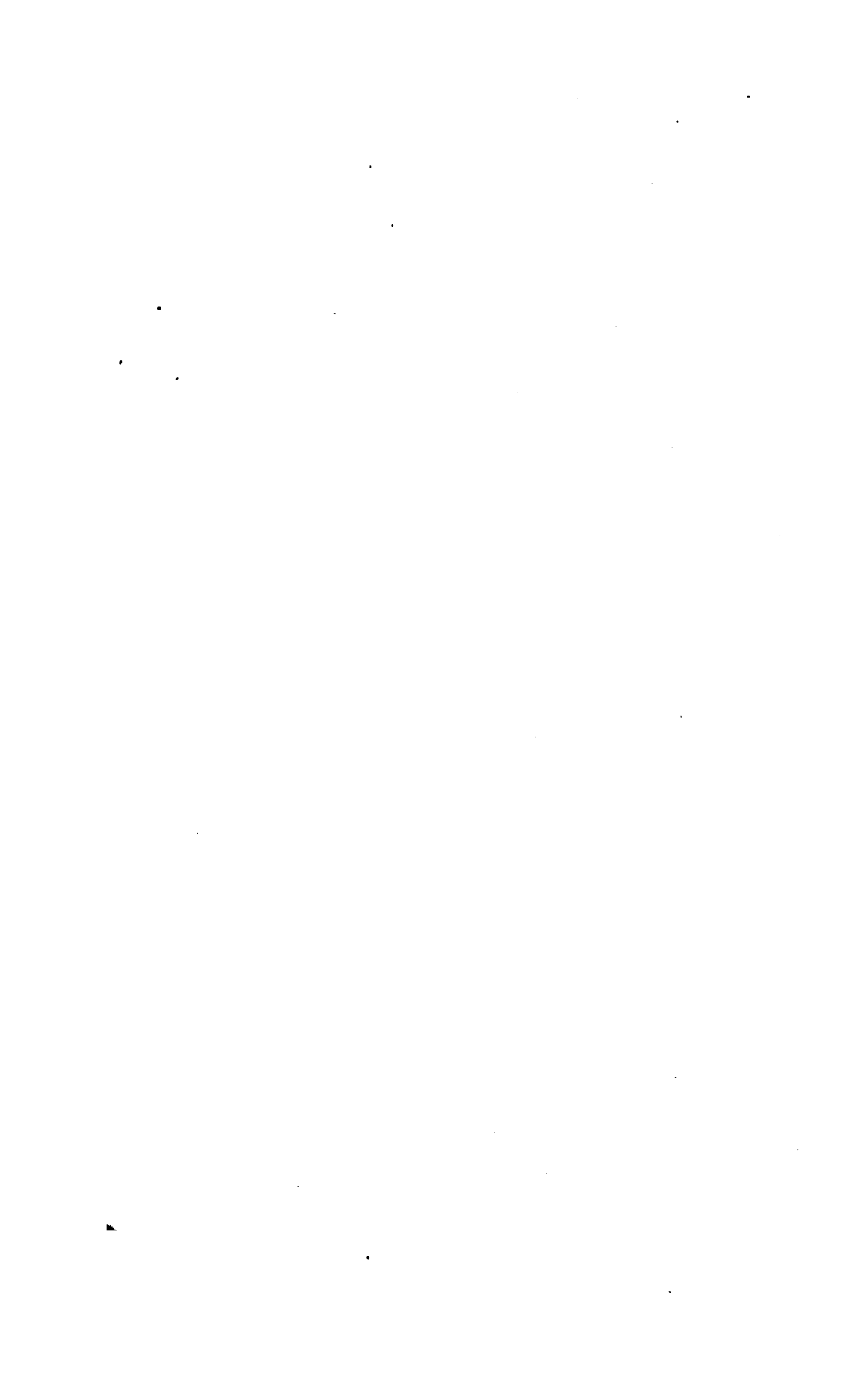
The magnesium in more than 100 determinations made on human blood serum (many of which were on pathological cases) has given figures varying from 0.8 to 3.8 mg. per 100 cc. of plasma. In the more strictly normal material, values from 1.6 to 3.5 mg. have been obtained.

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¹ For this reagent the modification described by Bloor (5) gives most satisfactory results.

² This standard is prepared from ammonium magnesium phosphate by dissolving 1.02 gm. of the pure salt in 100 cc. of N hydrochloric acid and diluting to a volume of 1 liter with distilled water; 1 cc. of this solution is equivalent to 0.10 mg. of magnesium. For the preparation of this dilute standard a portion of the strong solution is diluted 50 times with 0.1 N hydrochloric acid so that 5 cc. of the resulting solution will be equivalent to 0.01 mg. of magnesium.



A SYSTEM OF BLOOD ANALYSIS.

SUPPLEMENT I.

A SIMPLIFIED AND IMPROVED METHOD FOR DETERMINATION OF SUGAR.

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(Received for publication, January 26, 1920.)

In our system of blood analysis published a short time ago¹ we described a new method for the determination of sugar. In this method the sugar is oxidized by a weakly alkaline copper tartrate solution and the cuprous copper formed is estimated colorimetrically by the help of the phenol reagent of Folin and Denis. In this determination the errors due to creatine, creatinine, and uric acid are eliminated while a new source of error, namely that due to the so called phenols, is introduced. This last named error is certainly much smaller than the errors due to creatine, creatinine, and uric acid in other methods, so that in point of accuracy our method should be fully as good as any other known method. We could have eliminated any error due to the so called phenols by simply omitting the addition of sodium carbonate, because the phenol reagent reacts with cuprous copper in acid solution. Incidentally we should thus also eliminate the blank due to the alkaline copper tartrate. The process so obtained seemed to us less satisfactory, however, for the reason that the phenol reagent has a pronounced yellow color.² The matching of the colors in the colorimeter is rendered difficult and uncertain under such conditions, and would not be accurate except when the standard and the unknown are nearly of the same

¹ Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 106.

² The phenol reagent sometimes has a greenish color due to some reduction by organic dusts.

strength. We accordingly chose to destroy the surplus phenol reagent by the addition of sodium carbonate.

In the course of subsequent studies of the method we have found a process by which the disturbing effects of the phenols can be eliminated. To accomplish this purpose we have replaced the regular phenol reagent of Folin and Denis by a reagent which reacts with cuprous copper, in acid solution, yet gives no color with phenols. This new reagent is prepared as follows:

Transfer to a liter beaker 35 gm. of molybdic acid and 5 gm. of sodium tungstate. Add 200 cc. of 10 per cent sodium hydroxide and 200 cc. of water. Boil vigorously for 20 to 40 minutes so as to remove nearly the whole of the ammonia present in the molybdic acid.³ Cool, dilute to about 350 cc., and add 125 cc. of concentrated (85 per cent) phosphoric acid. Dilute to 500 cc.

It will be seen that the preparation of this reagent is much simpler than the preparation of the phenol reagent. The solution has none of the yellow color of the phenol reagent, yet gives an intense blue color with cuprous oxide. Since the reaction takes place in acid solution the blue color of the alkaline copper tartrate is also eliminated. The sodium tungstate contained in this reagent is added because there is sodium tungstate in our blood filtrates, and tungstates modify somewhat the shade of blue obtained in the reaction.

The alkaline copper solution has not been changed. Dissolve 40 gm. of pure anhydrous sodium carbonate in about 400 cc. of water and transfer to a liter flask. Add 7.5 gm. of tartaric acid, and when the latter has dissolved add 4.5 gm. of crystallized copper sulfate. Mix and make up to a volume of 1 liter. If the chemicals used are not pure a sediment of cuprous oxide may form in the course of 1 or 2 weeks. If this should happen, remove the clear supernatant reagent with a siphon, or filter through a good quality filter paper. Our reagent seems to keep indefinitely. To test for the absence of cuprous copper in the solution, transfer 2 cc. to a test-tube and add 2 cc. of the molybdate phosphate solution; the deep blue color of the copper should almost completely vanish. In order to forestall improper use of this reagent attention should be called to the fact that it contains

³ The molybdic acid we used was obtained from the Primos Company, Primos, Pa., and it contained considerable ammonia.

extremely little alkali, 2 cc. by titration (using the fading of the blue copper tartrate color as indicator), requiring only about 1.4 cc. of normal acid.

Standard Sugar Solutions.—Three standard sugar solutions should be on hand: (1) a stock solution, 1 per cent dextrose or invert sugar, preserved with xylene or toluene; (2) a solution containing 1 mg. of sugar per 10 cc. (5 cc. of the stock solution diluted to 500 cc.); (3) a solution containing 2 mg. of sugar per 10 cc. (5 cc. of the stock solution diluted to 250 cc.). The invert sugar solution has the advantage that it can be easily prepared from cane sugar, which is pure. The keeping quality of such solutions should be less good than those made from glucose, but we have encountered no trouble on that score. When good quality glucose is available, it is, of course, the one to use. The diluted solutions should be preserved with a little added toluene or xylene; it is probably better not to depend on such diluted solutions to keep for more than a month, but the stock solution should keep indefinitely.

It is a well known fact that the cuprous compounds produced by sugar in alkaline copper solutions show a marked tendency to be reoxidized to the cupric condition when exposed to air. "Most of us" are all too familiar with that fact in connection with ordinary sugar titrations done by overcautious students. That such reoxidation must occur to some extent in our colorimetric blood sugar determination is also undeniable. Without having made any direct experiments on the extent of such reoxidations we had satisfied ourselves that they do not contribute any material error in our sugar determinations. Check experiments with sugar solutions 50 per cent apart and heated 4, 6, and 8 minutes had given proportionate values. And in actual blood sugar determinations the values obtained were not changed by varying the heating time from 4 to 8 minutes. We had therefore no occasion to fear that material analytical errors could creep in because of the reoxidation.

At the last annual meeting of the American Association of Biological Chemists (Cincinnati, 1919), Benedict condemned our blood sugar method on the ground of excessive, inevitable, and uncontrollable reoxidations of cuprous oxide. He cited shaking experiments by means of which more than 60 per cent were made

to disappear. He also asserted that reoxidations are much more extensive in blood filtrates than in pure sugar solutions and therefore insisted that the blood sugar values obtained by our method must be too low. Vigorous shaking, as it happens, is also disastrous to the reaction between reducing sugar and alkaline picrates. Losses of 40 per cent can be secured by shaking (in 25 cc. flasks), and if agitation by an air current is substituted for shaking, nearly the whole of the sugar represented in a blood sugar determination by Benedict's method is lost. We cite these observations not as a criticism of Benedict's method but merely to show the grossly misleading character of shaking experiments.

It must be admitted nevertheless that we made something of an error in depending exclusively on indirect evidence on so important a point as the losses of cuprous oxide, and we gladly give Benedict credit for having compelled us to reexamine our method with reference to the effect of reoxidation. We have verified our earlier findings that analytical errors do not occur because of such reoxidation, but this is because all our test-tubes in which the oxidation of sugar takes place are of substantially the same diameter, 17 to 18 mm. on the inside. The oxidation in such tubes, when kept in a somewhat slanting position, may amount to as much as 20 per cent of the cuprous oxide formed, yet correct sugar values are obtained.

In order to get data on the reoxidation problem we have conducted the reduction in test-tubes of different internal diameters ranging from 4 to 23 mm. With test-tubes in a vertical position there is practically no loss of cuprous oxide by reoxidation until the diameter of the tube is at least 15 mm. At about 20 mm. or above the losses become astoundingly large. Currents having a somewhat similar effect as shaking must come into play in such tubes for the losses are not at all proportionate to the areas of the surface of solution. Shaking was of course excluded. The results of two series of experiments of the kind indicated are shown in Table I.

The remarkable fact about these reoxidations is that the percentage loss of copper so produced is not materially dependent on the amount of cuprous oxide involved. In a given tube the percentage loss is substantially the same whether one works with 0.1 or 0.4 mg. of sugar, whether with pure sugar solution or our blood

filtrate, and this is manifestly the reason why our original method has given uniformly concordant values. The results recorded in Table II show that the per cent loss of copper is almost independent of the amount of reduction involved. The reductions were made simultaneously in test-tubes of definite but widely different internal diameters.

TABLE I.

Relation Between Area of Surface of Solution and Loss of Cuprous Oxide by Reoxidation, Using 0.1 Mg. of Sugar.

Internal diameter of tube at level of surface of solution.	Colorimeter reading.	Loss.
mm.		per cent
4*	19.8	0
6	20	0
9	20	0 (?)
11	20	0 (?)
14	20, 20.5	0, 3
16	21.4, 22.5	7, 13
19	27.5, 29.5	27, 32
23	31, 32	36, 36

* In hydrogen atmosphere.

TABLE II.

Showing that Percentage Loss of Cuprous Oxide by Reoxidation Is not Materially Affected by Amount of Sugar Used for Reduction.

Amount of sugar used.	Colorimeter readings.		
	Constricted tube, 4 mm.	Open tube, 16 mm.	Open tube, 20 mm.
mg.			
0.1	20	22	28
0.2	20	21.8	
0.4	20	22	26
0.8	10	11	

In view of these findings it is necessary to be more precise than we had been in our description of the kind of test-tubes to be used. We prescribed test-tubes having the dimensions 200×20 mm. because ours were bought under those specifications, whereas in point of fact their internal diameter is only 17 to 18 mm. The subject is of sufficient importance to call for a special

tube by the use of which reoxidation is automatically excluded. This special blood sugar test-tube is shown in Fig. 1. The essential point to be observed in connection with it is, of course, that the surface of the alkaline mixture of sugar and copper shall reach the constricted part.

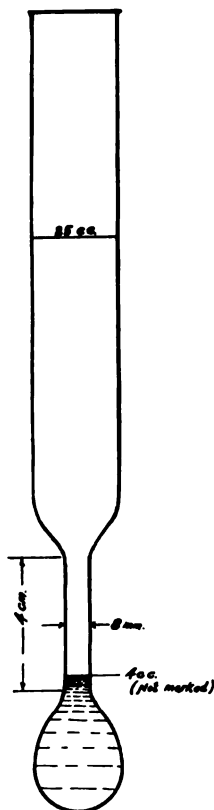


FIG. 1. Blood sugar tube.

Test-tubes of this sort, with and without graduation, are now made by The Emil Greiner Company, 55 Fulton Street, New York, and can also be obtained from Arthur H. Thomas Company, West Washington Square, Philadelphia. For the benefit of those who wish to make and graduate their own test-tubes we

suggest that a stem of about the size of a cherry stem should first be attached to the bottom of the test-tube. This stem is best produced by sealing on a piece of a broken test-tube and then drawing it out. If a good blast lamp is available, the whole process of making a tube does not take more than 2 or 3 minutes.

The blood sugar determination is now made as follows:

Transfer 2 cc. of the tungstic acid blood filtrate to a blood sugar test-tube, and to two other similar test-tubes (graduated at 25 cc.) add 2 cc. of standard sugar solution containing respectively 0.2 and 0.4 mg. of dextrose. To each tube add 2 cc. of the alkaline copper solution. The surface of the mixtures must now have reached the constricted part of the tube. If the bulb of the tube is too large for the volume (4 cc.) a little, but not more than 0.5 cc., of a diluted (1 : 1) alkaline copper solution may be added. If this does not suffice to bring the contents to the narrow part, the tube should be discarded. Test-tubes having so small a capacity that 4 cc. fills them above the neck should also be discarded. Transfer the tubes to a boiling water bath and heat for 6 minutes. Then transfer them to a cold water bath and let cool, without shaking, for 2 to 3 minutes. Add to each test-tube 2 cc. of the molybdate phosphate solution. The cuprous oxide dissolves rather slowly if the amount is large but the whole, up to the amount given by 0.8 mg. of dextrose, dissolves usually within 2 minutes. When the cuprous oxide is dissolved, dilute the resulting blue solutions to the 25 cc. mark, insert a rubber stopper, and mix. It is essential that adequate attention be given to this mixing because the greater part of the blue color is formed in the bulb of the tube.

The two standards given representing 0.2 and 0.4 mg. of glucose are adequate for practically all cases. They cover the range from about 70 to nearly 400 mg. of glucose per 100 cc. of blood.

It will be noted that in the process described we prescribe cooling of the alkaline cuprous oxide suspension before adding the phosphate molybdate solution. This cooling is not essential and, in case of one or two determinations only, may be omitted. In a large series of determinations it is probably best to use it. The important point is that the standard and the unknowns should not only be heated the same length of time but should also have

substantially the same temperature when the acid reagent is added. The maximum color develops faster in hot solutions; but if a reasonable uniformity of condition is maintained it makes no difference whether the color comparison is made at the end of 5 minutes or at the end of 1 hour.

TABLE III.

Comparison of Blood Sugar Values per 100 Cc. Obtained by Original and Revised Methods.

Source.	Revised method.	Original method.	
		Open tube.	Constricted tube.
	mg.	mg.	mg.
1* Human.	30	34	
2 "	61	65	
3 "	90	91	
4 "	99	93	
5 "	105	108	
6 "	90	98	
7 "	105	121	
8 "	121	129	125
9 "	134	130	130
10 "	131	131	131
11 "	200	210	218
12 "	224	218	228
13 Dog.	86	85	
14 "	84	85	

* Blood 24 hours old.

In our blood sugar method as we now have it reoxidations of the cuprous compounds are excluded; the blank due to the blue alkaline copper tartrate is eliminated, and, finally, the error due to the so called phenols in blood filtrates is removed. From a theoretical standpoint the method now appears to be without a flaw, and from the experimental standpoint it is rather more simple, inasmuch as the addition of sodium carbonate is omitted. The analytical figures in Table III show that the new process tends to give slightly lower values than the original method.

DISTRIBUTION OF THE BASIC NITROGEN IN PHASEOLIN.

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(Received for publication, January 14, 1920.)

Determination of the hydrolytic products of phaseolin, the chief protein of the navy bean, *Phaseolus vulgaris*, has been made by Osborne and Clapp (1). They did not find the amino-acid content essentially different from other vegetable globulins. It contained a sufficient quantity of the amino-acids known to be necessary for normal growth, with the possible exception of cystine. Nutrition experiments, however, by Osborne and Mendel (2), and McCollum, Simmonds, and Pitz (3) with the proteins of this bean met with little success.

TABLE I.
Basic Amino-Acids in Phaseolin.

Amino-acid.	Van Slyke method.	Absolute method, Osborne and Clapp (1).
	<i>per cent</i>	<i>per cent</i>
Cystine	0.84	Not determined.
Arginine	6.11	4.87
Histidine	3.32	2.62.
Lysine	7.88	4.58

To obtain more information for use in our nutrition experiments with the navy bean, an analysis of phaseolin by the Van Slyke (4) method was made. It will be noted that the results found by this method for the basic amino-acids were higher than those obtained by the direct method of Kossel and Patten (5), the value obtained for lysine being considerably greater. Sulfur determinations on the basic amino-acid fraction indicated the presence of 0.84 per cent of cystine. The accuracy of this method for the determination of cystine is uncertain particularly in cases where the per-

centage of sulfur in proteins is low, since a comparatively large correction has to be applied for the solubility of cystine phosphotungstate. The results of our analysis together with those obtained by Osborne and Clapp are summarized in Table I.

EXPERIMENTAL.

Analysis of Phaseolin by the Van Slyke Method.—Duplicate 3 gm. samples of phaseolin were used, each equivalent to 2.8476 gm. of moisture and ash-free protein and containing 15.99 per

TABLE II.

Analysis of Phaseolin by Van Slyke Method. Nitrogen Corrected for Solubility of Bases.

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N	0.0496	0.0495	10.90	10.88	10.89
Humin N adsorbed by lime. .	0.0079	0.0077	1.73	1.70	1.72
“ N in amyl alcohol extract	0.0008	0.0020	0.17	0.45	0.31
Cystine N	0.0027	0.0029	0.59	0.64	0.61
Arginine N	0.0545	0.0576	11.97	12.65	12.31
Histidine N	0.0260	0.0252	5.71	5.53	5.62
Lysine N	0.0427	0.0437	9.38	9.60	9.49
Amino N of filtrate	0.2470	0.2533	54.25	55.63	54.94
Non-amino N of filtrate	0.0209	0.0146	4.59	3.21	3.90
Total N regained	0.4521	0.4565	99.29	100.29	99.79

TABLE III.

Basic Amino-Acids in Phaseolin.

Amino-acid.	I	II	Average.
	per cent	per cent	per cent
Cystine	0.81	0.87	0.84
Arginine	5.94	6.28	6.11
Histidine	3.37	3.27	3.32
Lysine	7.79	7.97	7.88

cent or 0.4553 gm. of nitrogen. Each sample was hydrolyzed for 24 hours by boiling with 100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed by the

amyl alcohol-ether method (6). The results of these analyses are recorded in Tables II and III.

SUMMARY.

1. The basic amino-acids of phaseolin were determined by the Van Slyke method of analysis.

2. The percentage of lysine was found to be considerably higher than that obtained by the direct method of Kossel and Patten.

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STUDIES IN NUTRITION.*

II. THE RÔLE OF CYSTINE IN NUTRITION AS EXEMPLIFIED BY NUTRITION EXPERIMENTS WITH THE PROTEINS OF THE NAVY BEAN, PHASEOLUS VULGARIS.

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(Received for publication, January 14, 1920.)

A great advance was made in our knowledge of the principles of nutrition when it was shown that the nutritive value of proteins depends on the relative proportions of certain amino-acids in the protein molecule. Numerous experiments have demonstrated that the withdrawal or addition of certain amino-acids renders diets deficient or makes them complete for normal growth. It is well known that proteins which lack either lysine or tryptophane fail to promote the growth of animals. It has also been established that certain other amino-acids are necessary for maintenance and growth and that some of the amino-acids usually found in proteins are not essential.

Thus, Ackroyd and Hopkins (1) have shown that arginine and histidine are to some extent interchangeable. Totani (2) does not regard tyrosine as an essential amino-acid and suggests the possibility that phenylalanine may take its place in nutrition. Hopkins (3) has also shown that when both glutaminic and aspartic acids were removed from the products of hydrolysis of a biologically complete protein the resulting amino-acid residue was still adequate for normal growth. The theory that the nutritive value of a protein depends upon its amino-acid content encountered an apparent contradiction in the fact that phaseolin from the navy bean failed to promote growth, although it had been shown by Osborne and Clapp (4) that this protein contains all the known essential amino-acids.

* Read before The National Academy of Sciences, New Haven, 1919.

The nutritive value of phaseolin has been studied by Osborne and Mendel (5) who used the isolated protein in their experiments. The animals failed to grow and died in a relatively short time. McCollum, Simmonds, and Pitz (6) fed navy bean meal as the sole source of protein in the diet and their experiments also resulted in a failure to obtain growth. These authors attribute the failure of the navy bean to promote growth to the presence of hemicelluloses.

In our experiments where navy bean meal was used as the sole source of protein we could not detect any symptoms of tympanites although the animals failed to grow.

In making a study of the chemical analysis of phaseolin published by Osborne and Clapp (4) it seemed that all the known essential amino-acids were present in sufficient quantities to promote growth with the possible exception of cystine. An analysis of phaseolin (7) made by Van Slyke's method did not throw any further light on the subject and gave higher results for the basic amino-acids than those reported by Osborne and Clapp. This still left the question of a possible deficiency of cystine. It was evident that the percentage of cystine in phaseolin cannot be very high since this protein contains but approximately 0.3 per cent of sulfur. If all this belonged to cystine there would be only about 1.2 per cent of this amino-acid in phaseolin. It is probable, however, that only about one-third of the total sulfur in phaseolin represents cystine, as shown by Osborne (8) who determined the percentage of total sulfur liberated when a number of different proteins were boiled with potassium hydroxide. It was assumed that approximately two-thirds of the cystine sulfur of proteins was thus liberated as hydrogen sulfide. This assumption was based on experiments with pure cystine from which approximately two-thirds of the total sulfur was liberated as hydrogen sulfide when treated in the same manner. It is not known in what form the non-cystine sulfur exists in proteins.

In view of the above facts it appeared that the failure of phaseolin to promote growth might be due to a deficiency in cystine. 2 per cent of cystine, prepared from wool, was therefore added to the phaseolin and 18 to 20 per cent of this mixture was used in a diet together with the essential non-protein ingredients. The animals placed on this diet maintained their weight for a con-

siderable time and grew slightly but the results were far from satisfactory. While the addition of cystine had improved the nutritive value of phaseolin the failure to obtain normal growth was still an unsolved problem.

The question of the digestibility of phaseolin was next considered, since metabolism experiments (9, 10) had shown that the nitrogen of the navy bean was not well assimilated. Phaseolin was therefore subjected to digestion with trypsin *in vitro*. The usual procedure was followed, the mixture being made slightly alkaline with sodium carbonate and the digestion carried out at 37°C. To prevent bacterial growth, the phaseolin was heated at 100°C. in air and 0.1 per cent of sodium benzoate was added to the distilled water used in the digestion. When incubation was complete the sodium carbonate was neutralized with hydrochloric acid and the solution evaporated to dryness on a steam bath. The residue was ground to a powder and 2 per cent of cystine was added. When this digested phaseolin, supplemented with cystine, was used as the sole source of protein in the diet, the rats grew normally. There were then several factors to consider. Was the success obtained due to the digestion with trypsin, or the alkaline action of the sodium carbonate, or the heating during the evaporation? It seemed improbable that the sodium benzoate was one of the factors, other than aiding in maintaining sterility. Bacterial counts¹ were made before and after incubation by making cultures on agar. The bacterial content was so low that fermentation from this source was eliminated as a factor. Experiments were made in which the trypsin, sodium carbonate, and sodium benzoate were eliminated one by one, and normal growth was still obtained when the phaseolin was simply suspended in water, boiled for 30 minutes, and the mixture evaporated to dryness, 2 per cent of cystine being added to the residue before preparing the diet. A ration containing phaseolin which had been heated with water, but to which no cystine had been added, was also tried and slow growth was obtained. When cystine was added to this ration the rate of growth became normal. The success of the experiments, therefore, depended on two factors. *Phaseolin is rendered a more efficient food by heating it with water. It must also be supplemented by cystine to obtain normal growth.*

¹ The bacterial counts were made by Miss R. B. Edmondson of the Microbiological Laboratory, Bureau of Chemistry.

It is difficult to explain why heating phaseolin with water renders it more nutritious. This process may cause some molecular rearrangement to occur in the very complex and probably labile protein molecule, thereby making it more readily digestible or the phaseolin may possess a slight toxicity which is destroyed by heating with water.

It now seemed important to ascertain whether or not navy bean meal could be rendered efficient for normal growth by the process mentioned above. The cooked meal had been tried in our earlier experiments but while the rats maintained their weight for a considerable time only slight growth was obtained. The bean meal yielded to treatment in the same manner as phaseolin. When cooked, dried, and supplemented with cystine, it produced normal growth when used as the sole source of protein in the diet.

The importance of cystine as an ingredient of a complete diet has been shown by a number of investigators. Osborne and Mendel (11) have shown that, when casein is supplemented with cystine, 9 per cent of the casein is as effective as 15 per cent of casein alone. This percentage they found to be necessary for normal growth in diets of high calorific value.

Abderhalden (12) conducted nutrition experiments with hydrolyzed proteins from which he had attempted to remove the cystine. He did not reach any definite conclusion, although he states that cystine is apparently necessary. Hopkins and his coworkers (1, 2, 3), in their nutrition experiments with hydrolyzed proteins, always added cystine, since this amino-acid is partly decomposed by heating with acids during the process of hydrolysis. Mitchell (13) and Geiling (14) also added cystine to the diets used in their experiments with white mice when feeding amino-acid mixtures. Lewis (15), experimenting on dogs, showed that if small quantities of cystine were added to diets low in protein the nitrogen elimination of the animals diminished, whereas an equivalent quantity of nitrogen in the form of tyrosine or glycine added to the diet did not diminish the quantity of nitrogen eliminated. It is evident that Lewis was experimenting with diets in which cystine was a limiting factor. Daniels and Rich (16) attempted to substitute inorganic sulfates for cystine but without success.

Casein contains 0.8 per cent of sulfur but Osborne (8) has shown that approximately only 0.15 per cent of the casein is cystine sul-

fur. McCollum, Simmonds, and Pitz (6) found that casein was not a very effective supplement to navy bean proteins. Their observation is now readily interpreted when it is considered that they were supplementing the navy bean protein, which is low in cystine, with casein which also is low in cystine. It seems probable that cystine was the limiting factor in this mixture of proteins. These facts lend a new interest to the experiments of Osborne (8) in which he attempted to ascertain the proportion of cystine sulfur to the total sulfur in a number of proteins. The proteins were boiled with potassium hydroxide and the sulfur liberated as hydrogen sulfide was multiplied by three-halves to obtain the quantity of cystine sulfur in the proteins. The results indicated that the sulfur in the form of cystine varied from approximately 20 to 75 per cent of the total sulfur in the fifteen vegetable and animal proteins examined (17). He noted that casein and phaseolin, which become more efficient when supplemented with cystine, both yield but small quantities of hydrogen sulfide when boiled with alkali. On the other hand such proteins as ovalbumin, fibrin, ovovitellin, and edestin, which are known to be efficient proteins, yield a relatively large quantity of hydrogen sulfide.

The work herein described was reported in a preliminary paper read before the Society of Biological Chemists at the Baltimore meeting in April, 1919. At that time Osborne and Mendel informed us that they had conducted further experiments on the nutritive value of phaseolin but the results of this work had not been published. They also found that the nutritive value of phaseolin was increased by cooking. Furthermore, they found that the protein extracted from the navy bean by sodium hydroxide and reprecipitated by neutralization was more effective than phaseolin prepared by extraction with sodium chloride and precipitated by dialysis. Osborne and Mendel very kindly placed the results of their unpublished work at our disposal and some of the curves shown in this paper are taken from their experiments.

Experiments with Raw and Cooked Phaseolin.—This work was done by Osborne and Mendel who found that when raw phaseolin was the sole source of protein in an otherwise adequate diet the rats declined in weight rapidly and some of them died. When the phaseolin was replaced by casein the growth became normal. By alternating phaseolin with casein in the diet the rats could be

made to lose weight or grow normally. Phaseolin which had been cooked for a short time in water served to maintain weight but produced practically no growth. In all these experiments the water-soluble vitamins and salts were supplied by protein-free milk and the fat-soluble vitamins by butter fat. The curves are shown in Chart 1.

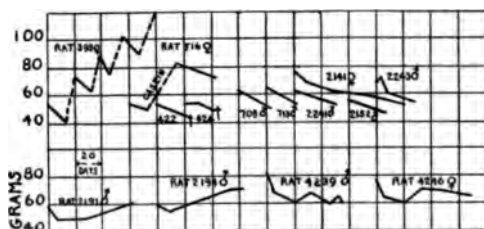


CHART 1. The upper set of curves shows the failure of raw phaseolin to maintain the weight of white rats. In the curves for Rat 393 ♀ and Rat 516 ♀ the solid lines of the curves show the effects of a diet in which phaseolin was the sole source of protein. The broken portions of the curves show the effect of replacing the phaseolin with casein. The daggers indicate that the rats died.

The lower set of curves shows the effect of cooking the phaseolin before preparing the diet. It will be seen that the rats were almost able to maintain their weights for a considerable time but could not grow. The phaseolin was stirred into distilled water and cooked for 5 minutes after the water had been heated to the boiling point. It was then filtered off and dried. The curves in this chart represent the results of unpublished experiments by Osborne and Mendel. The composition of the diets follows.

	Uncooked. gm.	Cooked. gm.
Phaseolin (or casein).....	18	18
Protein-free milk.....	28	28
Starch.....	28	28
Butter fat.....		18
Lard.....	26	8
	<hr/> 100	<hr/> 100

Experiments with Phaseolin Supplemented with Cystine.—The raw phaseolin² used in these experiments was supplemented with 2

² The phaseolin and cystine used in these experiments were prepared by Mr. C. E. F. Gersdorff of the Protein Investigation Laboratory, Bureau of Chemistry.

per cent of cystine and this mixture was used as the sole source of protein in an otherwise adequate diet. The addition of cystine to the raw phaseolin enabled the rats to grow slowly. A mixture of cooked phaseolin supplemented with cystine at once improved the growth. The replacement of the raw phaseolin supplemented with cystine by casein after the rats had grown but very little for some time also caused a marked improvement in the rate of growth. The results of these experiments are shown in Chart 2.

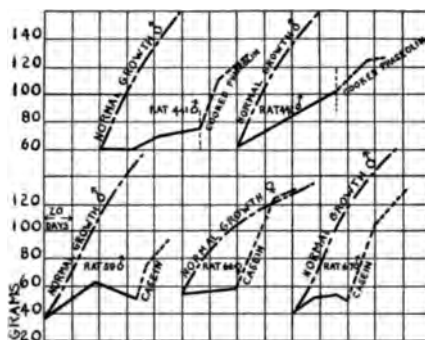


CHART 2. The upper set of curves shows that raw phaseolin supplemented with cystine enabled the rats to grow slowly. During the period indicated by the dotted portion of these curves the rats received cooked phaseolin supplemented with cystine. A marked improvement in the rate of growth occurred at once when the raw phaseolin was replaced by the cooked protein.

In the experiments represented by the lower set of curves the rats received raw phaseolin supplemented with cystine. During the second period, shown by the broken lines, the animals received casein instead of the phaseolin and cystine.

The composition of the diet follows.

	gm.
Phaseolin.....	17.64
Cystine.....	0.36
Protein-free milk.....	28.00
Starch.....	21.00
Agar.....	5.00
Butter fat.....	18.00
Lard.....	10.00
	<hr/>
	100.00

Experiments with Cooked Phaseolin Supplemented with Cystine.—A number of experiments were made with phaseolin cooked under various conditions and supplemented with cystine. In some cases the phaseolin was predigested with trypsin and cooked before adding cystine. Some of the diets contained sodium benzoate which had been used to keep the solution sterile during digestion with trypsin. In all cases the rats grew at a normal rate when the phaseolin was cooked, whether or not it had been predigested, if finally supplemented with cystine. In two experiments the animals received cooked phaseolin for some time without the addition of cystine. The rate of growth was far below normal but became normal when cystine was added to the diet. The results of these experiments are shown in Chart 3.

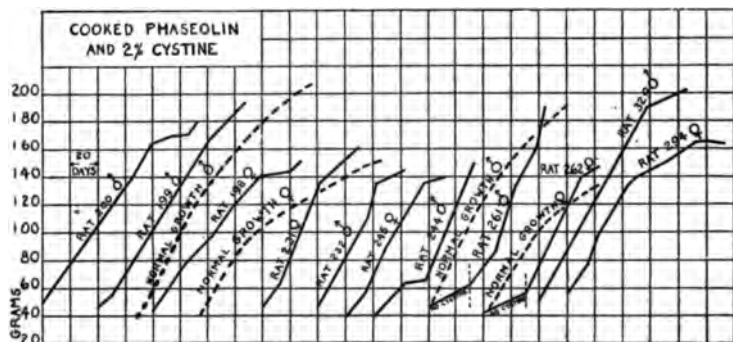


CHART 3. The curves in this chart show that normal growth was obtained on diets containing cooked phaseolin supplemented with cystine. The protein given to Rats 198 ♀, 199 ♂, and 200 ♂ was treated by suspending 12.5 gm. of phaseolin in distilled water to each 100 cc. of which had been added 0.5 gm. of sodium carbonate, 0.1 gm. of sodium benzoate, and 0.05 gm. of trypsin. This mixture was incubated for 5 hours at 37°C., heated to boiling, neutralized with hydrochloric acid, and evaporated to dryness on a steam bath. The residue was ground to a powder and mixed with 2 per cent of cystine. The phaseolin fed to Rats 231 ♀ and 232 ♂ was treated in the same manner but the trypsin was omitted. The phaseolin given to Rats 244 ♂ and 245 ♀ received similar treatment but without the addition of sodium carbonate or trypsin. The neutralization with hydrochloric acid was also omitted. The phaseolin fed to Rats 329 ♂ and 294 ♀ was simply boiled in distilled water for 30 minutes. The mixture was then evaporated to dryness on a steam bath. The residue was ground to a powder and supplemented with 2 per cent of cystine. Rats 261 ♂ and 262 ♀

during the first stage were placed on a diet containing phaseolin cooked in water. The second stage shows the remarkable improvement in the rate of growth due to the addition of 2 per cent of cystine to the cooked phaseolin.

The composition of the diet modified as stated above follows.

Phaseolin (cooked, etc.)	19.60
Cystine	0.40
Protein-free milk	28.00
Starch	22.00
Agar	5.00
Butter fat	18.00
Lard	7.00
	<hr/>
	100.00

Experiments with Navy Bean Meal.—Osborne and Mendel found that rats fed on raw bean meal declined rapidly in weight and soon died. When the bean meal was cooked slight growth was ob-

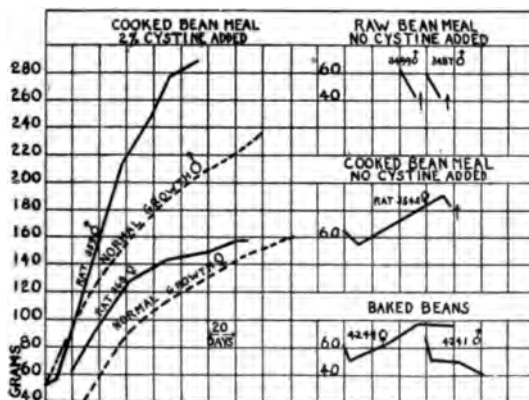


CHART 4. Rats 359 ♂ and 368 ♀ were fed on a diet containing cooked navy bean meal supplemented with cystine equivalent to 2 per cent of the protein. The bean meal was cooked with distilled water for 3 hours. The mixture was then evaporated to dryness and the residue ground to a powder to which cystine was added before preparing the diet. The excellent growth obtained shows that cooked navy beans, supplemented with cystine, furnished an adequate protein mixture.

The following data are taken from results obtained by Osborne and Mendel.

Rats 3499 ♂ and 3487 ♂ were fed on a diet in which the proteins were derived from raw navy bean meal. The curves show clearly the inadequacy

of this diet. Rat 3545 ♀ was fed on a diet in which the protein was furnished by cooked bean meal. It is evident that cooking the bean meal causes an improvement comparable to that resulting from cooking phaseolin. Rats 4244 ♀ and 4241 ♂ were fed on a diet in which the protein was supplied by beans baked without the addition of pork. It is evident that the proteins of the navy bean must be cooked and supplemented with cystine before they can become adequate for normal growth.

The composition of these diets follows.

	Rats 359♂, 368♀.	Rats 3499♂, 3487♂.	Rats 4244♀, 4241♂.	Rat 3545♀.
	gm.	gm.	gm.	gm.
Cooked navy bean meal.....	71.64			
Raw navy bean meal.....		35		54
Baked beans.....			72	
Corn gluten.....		19		
Cystine.....	0.36			
Salt Mixture IV*.....	4.00		3	
Protein-free milk.....		21		22
Butter fat.....	15.00	18	18	18
Lard.....	9.00	.7	7	6
	100.00	100	100	100

* For composition of salt mixture see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374.

tained for some time. Baked beans showed no greater efficiency than cooked bean meal. We found that cooked navy bean meal supplemented with cystine equivalent to 2 per cent of its protein produced normal growth. This diet contained butter fat to furnish fat-soluble vitamins. The salts were furnished by an artificial salt mixture and the beans supplied sufficient water-soluble vitamins. The curves are shown in Chart 4.

Similar experiments are being made on the proteins of other leguminous seeds and the results obtained will be reported in future publications.

SUMMARY.

1. Cystine has been shown to be essential for normal growth.
2. Phaseolin, the principal protein of the navy bean, is rendered a more efficient food by heating with water.
3. Cooked phaseolin or cooked navy bean meal when supplemented with cystine furnished adequate protein for normal growth.

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STUDIES IN NUTRITION.

III. THE NUTRITIVE VALUE OF COMMERCIAL CORN GLUTEN MEAL.

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The fact that corn, *Zea mays*, is the most abundant cereal grown in the United States has led a number of investigators to study the nutritive value of its proteins. Among such studies are those made by Osborne and Mendel (1, 2, 3, 4), McCollum, Simmonds, and Pitz (5), Hart, Halpin, and McCollum (6), and Hogan (7, 8). These researches have contributed greatly to our present knowledge of the nutritive value of corn proteins.

Corn is known to contain several different proteins (9, 10) and the approximate distribution of these in the kernels of a sample of corn containing a relatively high percentage of proteins is shown in the following table which is taken from a publication by Osborne and Mendel (2).

	<i>Per cent of protein.</i>
Globulins + albumins + "proteoses"	21.9
Zein	41.4
Maize glutelin	30.8
Insoluble in alkali	5.9
	<hr/> 100.0

Zein, which constitutes approximately from 41 to 52 per cent of the proteins in corn, lacks lysine and tryptophane, which are essential amino-acids for the growth of animals. It also contains but small quantities of the basic amino-acids which are also essential in a complete diet. Corn glutelin, which is the next most abundant protein, contains all the essential amino-acids which zein lacks and promotes normal growth. Osborne and Mendel

(11) also state that considerable growth can be obtained from corn embryos as the sole source of protein, likewise with a mixture of corn gluten and corn embryo when a sufficient quantity of the latter is present. We have, therefore, in corn a mixture of efficient and deficient proteins.

The chief difficulties in conducting nutrition experiments with ground whole corn as the sole source of protein is due to its relatively low percentage of protein. When the other essential components of the diet, namely, an adequate inorganic salt mixture, butter fat, and lard, are added, the percentage of protein is too low to expect normal growth.

The only abundant protein concentrates of corn on the market are commercial gluten meal and gluten feed. The gluten meal is obtained as a by-product in the manufacture of starch, dextrin, and syrup from corn. Approximately 600,000 to 700,000 tons of gluten meal and gluten feed are produced annually. The corn is steeped in water containing sulfur dioxide in order to soften it and is then ground under water and the mixture is strained through sieves to remove hulls, tip caps, and the embryos. The water containing the suspended starch and protein is passed over long troughs in which most of the starch settles. The protein passing out of the trough is filter-pressed and dried at a low temperature. This dried mixture is known commercially as gluten meal and is used extensively for making mixed cattle feeds. Gluten feed is made in the same manner but contains the residue obtained by evaporation of the steep water.

Several attempts have previously been made to grow white rats on corn gluten, prepared in various ways, as the sole source of protein. Osborne and Mendel have described nutrition experiments with gluten meal (2, 4). This gluten meal contained about 45 per cent of proteins. When this was the sole source of protein in the diet the rats maintained weight but made very little growth during an experiment which was continued for more than a year. The authors state that with this gluten meal (2) "more than very slow growth is impossible." When a part of the gluten meal was replaced by casein or lactalbumin normal growth was obtained. The inorganic salts and water-soluble vitamins were furnished by protein-free milk. The authors conclude their paper with the following statement: "It is perhaps not too utopian to expect that the day may come when amino-acid concentrates may serve to render perfect the mixtures of proteins in a fodder like maize or its commercial by-products." In a later publication (11) the same authors state that they have never yet been able to feed

a sufficient quantity of the proteins of the maize concentrate to effect normal growth although there is evidence that all the essential amino-acids are present in some part of the seed. They also state:

"Our experiments indicate that it ought to be possible to make an animal grow on a diet in which the maize kernel is the sole source of protein, provided a preparation of total proteins could be obtained which would permit feeding them in sufficient quantity so that enough of those amino-acids which are present in certain of the proteins and not in others would be available to meet the minimum nutritive requirements of the organism."

McCollum, Simmonds, and Pitz (5) have also attempted to grow rats on a diet containing various mixtures of separate parts of the corn kernel but were not successful in obtaining normal growth.

Hogan has made extensive studies of the nutritive value of corn and its proteins. In one of his experiments (7) he fed a mixture of corn and commercial corn gluten meal as the sole source of protein in the diet and obtained surprisingly good growth. He states that corn gluten seemed more efficient than either egg white or dried blood as a supplement to corn but adds that it is possible that this result should be confirmed by a larger number of experiments before the fact can be considered established. In a later experiment (8) he attempted to grow rats on a corn protein concentrate which he prepared himself in order that the embryo, which is absent in commercial corn gluten meal, might be present, but the attempts were not successful and some of the rats died relatively soon. In these experiments the percentages of corn proteins in the diet were only 9 or 12 per cent so that no definite conclusions can be drawn as to the value of the protein concentrate.

The purpose of our experiments with commercial gluten meal was not only to determine its nutritive value but also to endeavor to find some cheap concentrate that could be used to supplement the proteins of the gluten since zein, which constitutes approximately one-half of these proteins, is deficient in lysine and tryptophane. As will be seen in the description of the experiments given below this could be accomplished by the addition of coconut press-cake to the gluten meal. Normal growth was also obtained when a mixture of ground whole corn and gluten meal furnished the sole source of proteins in the diet. This diet contained 21 per cent of corn proteins.

Experiments with Gluten Meal as the Sole Source of Protein.—A diet was prepared which contained 53 per cent of gluten meal. This is equivalent to 19.4 per cent of protein. To this were added

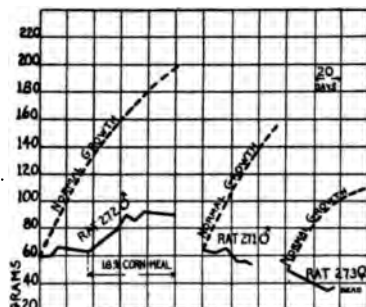


CHART 1. This chart shows the failure of rats to grow when gluten meal was the sole source of protein in the diet and no source of water-soluble vitamine was added. Rat 273 ♀ died in 36 days and the experiment was discontinued with Rat 271 ♂ at the end of that time, while 18 per cent of whole, ground, yellow corn replaced an equivalent amount of starch in the diet of Rat 272 ♂. This caused considerable growth for a short time; then growth ceased again. It is probable that Rat 272 ♂ was permanently injured by lack of water-soluble vitamine before the corn-meal was added. The composition of the diet before the adding of the ground corn was as follows:

	gm.	
Gluten meal.....	53 (Equivalent to 19.4 gm. of protein.)	
Salt mixture*.....	4	
Starch.....	18	
Butter fat.....	18	
Lard.....	7	
	<hr/>	
	100	

* The composition of the salt mixture was as follows:

	gm.		gm.
CaCO ₃	134.8	Citric Acid + H ₂ O...	111.1
MgCO ₃	24.2	Fe citrate + 1½ H ₂ O.	6.34
Na ₂ CO ₃	34.2	KI.....	0.020
K ₂ CO ₃	141.3	MnSO ₄	0.079
H ₃ PO ₄	103.2	NaF.....	0.248
HCl.....	53.4	K ₂ Al ₂ (SO ₄) ₄	0.0245
H ₂ SO ₄	9.2		

Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374.

a suitable salt mixture, starch, butter fat, and lard. Three rats were placed on this diet but none of them made any appreciable growth and one died at the end of 36 days. Rat 272 maintained its weight for 36 days and at the end of that time the 18 per cent of starch was replaced by whole, ground, yellow corn. Considerable growth took place but the rate was below normal and soon ceased.

There were two factors either of which might be responsible for failure of the rats to grow on the diet consisting of gluten meal as the sole source of protein. It was not known whether or not the gluten meal furnished proteins adequate for normal growth and if the necessary water-soluble vitamine was present. This accessory, which is necessary for growth, goes into the steep water. Since this was not incorporated in the gluten meal used in our experiments, it seemed probable that the failure to obtain growth was due to the lack of water-soluble vitamine. The curves obtained in these experiments are shown in Chart 1.

Experiments with Gluten Meal and Brewers' Yeast.—The diet used in these experiments contained 53 per cent of gluten meal and 3 per cent of dried brewers' yeast together with a salt mixture, starch, butter fat, and lard. Since brewers' yeast has been shown to contain an abundance of water-soluble vitamine (12) any failure to grow could not be attributed to the lack of this dietary essential. Two rats were placed on this diet and as is shown by the curves in Chart 2 the rate of growth was above normal which was surprising in view of the results obtained by other investigators. It is very evident that the yeast furnished an adequate supply of water-soluble vitamine. Since yeast also contains proteins adequate for normal growth (13), the success obtained in this experiment might be partly due to the proteins of the yeast although it seemed improbable that the small quantity of proteins contributed by 3 per cent of yeast could have much effect on the rate of growth.

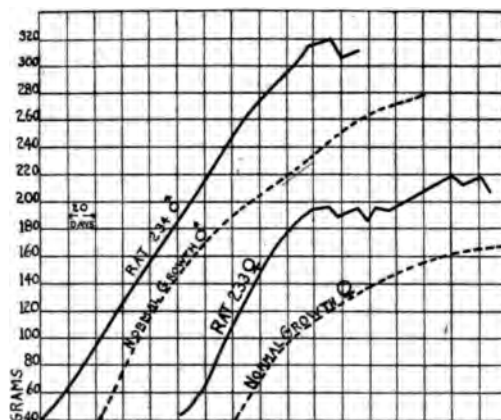


CHART 2. This shows excellent growth when gluten meal was the sole source of protein and the water-soluble vitamine was furnished by 3 per cent of dried brewers' yeast. With this exception the diet was similar to the one on which rats failed to grow (Chart 1). It is apparent that gluten meal contains a mixture of proteins adequate for normal growth. This contention is also borne out by the results shown by the curves in Chart 3 in which the water-soluble vitamine was furnished by 18 per cent of whole, yellow corn-meal.

The composition of the diet was as follows:

	gm.
Gluten meal.....	53 (Equivalent to 19.4 gm. of protein.)
Salt mixture.....	4
Starch.....	15
Dried brewers' yeast...	3
Butter fat.....	18
Lard.....	7
	<hr/>
	100

Experiments with a Mixture of Gluten Meal and Corn-Meal as the Sole Source of Protein and Water-Soluble Vitamine.—This diet contained 53 per cent of gluten meal and 18 per cent of yellow corn-meal made from the whole corn. Two rats were placed on this diet and their growth was remarkably good, the rate of growth of the female being normal. These results confirmed those obtained by Hogan (7, 8) on a similar diet and show that corn proteins of sufficient concentration in the diet are adequate for normal growth. It is also evident that 18 per cent of corn-meal

furnishes an adequate supply of water-soluble vitamine. It is probable, however, that the vitamine content of this diet was very near the minimum quantity required. The curves obtained in these experiments are shown in Chart 3.

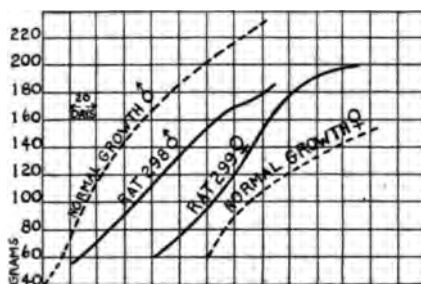


CHART 3. The curves in this chart show that normal growth can be obtained from a diet in which a mixture of gluten meal and ground corn furnishes the sole source of protein and water-soluble vitamine. The gluten meal contributed 19.4 per cent of protein in the diet while the ground corn contributed only 1.5 per cent. The water-soluble vitamine contributed by the ground corn was probably near the minimum requirement of this food accessory. The composition of the diet was as follows:

	gm.	
Gluten meal.....	53	(Equivalent to 19.4 gm. of protein.)
Salt mixture.....	4	
Whole ground corn.....	18	(Equivalent to 1.5 gm. of protein.)
Butter fat.....	18	
Lard.....	7	
	<hr/>	
	100	

Experiments with Mixtures of Gluten Meal and Coconut Press-Cake.—Although normal growth can be obtained from a diet in which concentrated corn proteins furnish the sole source of proteins, such a diet is probably not economical because approximately one-half of these proteins are deficient in lysine and tryptophane. It therefore seemed desirable to find a cheap protein concentrate which when mixed with gluten meal would contribute sufficient quantities of lysine and tryptophane to constitute an efficient mixture of proteins. Our previous work on the nutritive value of the proteins of coconut press-cake (14) suggested that this substance might be used advantageously for this pur-

pose. A diet was, therefore, prepared in which 25 per cent of corn gluten and 25 per cent of coconut press-cake furnished the sole source of protein. This diet contained 14 per cent of protein ($N \times 6.25$) of which approximately 9 per cent was furnished by corn gluten and 5 per cent by coconut press-cake. The rest of the diet consisted of a salt mixture, starch, butter fat, and lard.

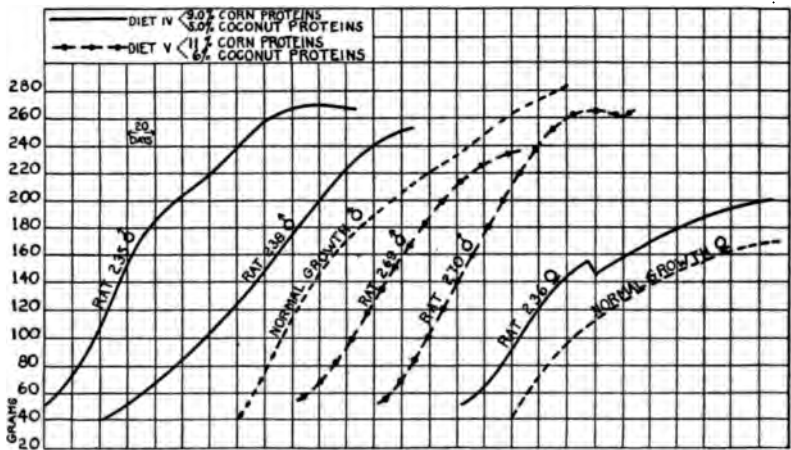


CHART 4. These curves show that excellent growth can be obtained when a mixture of gluten meal and coconut press-cake furnishes the sole source of protein and water-soluble vitamins in the diet. The high efficiency of this mixture is indicated by the results obtained on Diet IV which contained but 14 per cent of proteins of which only 5 per cent was furnished by the coconut press-cake. This indicates the value of coconut press-cake as a supplement to corn proteins. The compositions of Diets IV and V are as follows:

	Diet IV.	Diet V.
	gm.	gm.
Gluten meal	25	30
Coconut press-cake	25	31
Salt mixture	4	4
Starch	21	10
Butter fat	10	18
Lard	15	7
Total	100	100

The rats (Nos. 235, 238, and 236) fed on this diet grew normally, indicating an efficient mixture of proteins. It is also evident that 25 per cent of coconut press-cake furnished sufficient water-soluble vitamine for normal growth. A second diet containing 30 per cent of gluten meal and 31 per cent of coconut press-cake also gave excellent results. The growth obtained in these experiments is shown by the curves in Chart 4. Hence, it seems practical and economical to supplement gluten meal with coconut press-cake both for the purpose of supplementing the proteins of gluten meal and adding the water-soluble vitamine which gluten meal lacks.

SUMMARY.

1. Commercial corn gluten meal supplemented by dried brewers' yeast, whole, ground, yellow corn, or coconut press-cake furnishes the necessary protein for normal growth.
2. 18 per cent of whole, ground, yellow corn-meal furnishes sufficient water-soluble vitamine for normal growth.

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THE EQUILIBRIUM BETWEEN OXYGEN AND CARBONIC ACID IN BLOOD.

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I.

After many years of uncertainty, a very high degree of probability seems to have been established for the hypothesis that the absorption of oxygen by blood depends upon a chemical combination between oxygen and hemoglobin, $\text{Hb} + \text{O}_2 = \text{HbO}_2$, in which the molecular weight of hemoglobin has the lowest possible value, so that 1 atom of iron corresponds to 1 molecule of oxygen in the compound oxyhemoglobin.

This chemical equilibrium has been very thoroughly studied by Barcroft and his associates (1). As a result of their investigations it is known that pure solutions of hemoglobin combine with oxygen in such a manner that the requirements of the simple mass law equation

$$k = \frac{[\text{Hb}] \cdot [\text{O}_2]}{[\text{HbO}_2]} \quad (1)$$

are fulfilled. But when electrolytes are also present in the solution this relation is modified. Under such circumstances Barcroft has found that an equation developed by Hill¹

$$k = \frac{[\text{Hb}] \cdot [\text{O}_2]^n}{[\text{HbO}_2]} \quad (2)$$

expresses the conditions. In this equation, however, the values of n and k vary with the nature and concentration of the electrolytes. For human blood the value of n is always approxi-

¹ Barcroft (1), p. 60.

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mately 2.5, and the expression becomes

$$k = \frac{[\text{Hb}] \cdot [\text{O}_2]^{2.5}}{[\text{HbO}_2]} \quad (3)$$

The theoretical significance of this expression, in spite of many cogent arguments, remains somewhat obscure. This question will be further considered below.

With constant temperature, the principal variation in the value of k in human blood depends upon variations of the concentration of free carbonic acid, a quantity which is determined

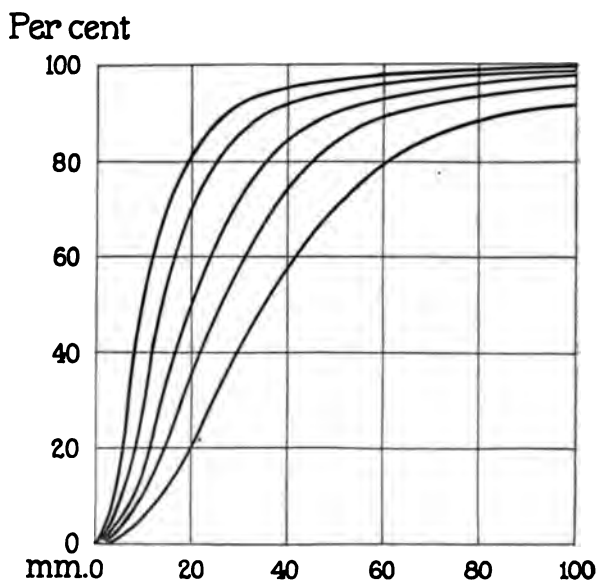


FIG. 1. Oxygen dissociation curves of Barcroft's blood, reading from left to right, exposed to 0, 3, 20, 40, and 90 mm. of CO_2 . Ordinates are percentage saturation; abscissæ oxygen pressures in mm. of Hg.

by, and proportional to, the tension of carbon dioxide. This is the phenomenon discovered by Bohr, Hasselbalch, and Krogh (2). These conditions are illustrated by dissociation curves of Barcroft's blood² (Fig. 1).

For the curves of Fig. 1 the values of k (corresponding to $\frac{1}{K}$

² Barcroft (1), p. 65.

in Barcroft's notation) are as follows:

CO ₂ , mm.....	0	3	20	40	90
k.....	388	770	1,980	3,430	7,420

It may readily be seen, when the large variations in the values of k are noted, that we have no great assurance that the value of k for a single tension of carbon dioxide, *i.e.* for a single curve, is not affected with secondary variations, especially near the ends of the curves.

The simplicity of the mathematical expression also suggests that it should be possible to express the value of k in terms of $[\text{CO}_2]$, the tension of carbon dioxide, and thus to obtain the equation of the surface whose axes are percentage saturation with oxygen, oxygen pressure, and carbon dioxide pressure respectively. This expression turns out to be as follows:

$$\frac{[\text{CO}_2] + 7.7}{0.014} = \frac{[\text{Hb}] \cdot [\text{O}_2]^{2.5}}{[\text{HbO}_2]} \quad (4)$$

For, putting $k = \frac{[\text{CO}_2] + 7.7}{0.014}$ we get the following values:

[CO ₂], mm.....	0	3	20	40	90
k.....	550	764	1,980	3,410	6,980

These values correspond to those of Barcroft within 1 per cent throughout the greater part of the range of carbon dioxide tension, and differ sensibly only in case carbonic acid is entirely absent, a peculiarity which, on theoretical grounds, is to be expected.

When Equation 4 is written

$$\frac{[\text{CO}_2] + 7.7}{0.014 \cdot [\text{O}_2]^{2.5}} = \frac{[\text{Hb}]}{[\text{HbO}_2]}$$

it is evident, from general thermodynamical principles, since increasing the concentration of carbon dioxide results in an increase of the concentration of oxygen, that increasing the tension of oxygen must likewise increase the tension of carbon dioxide. This conclusion, as far as I can see, could fail only in case there were present something resembling a ratchet mechanism, by which the carbon dioxide could act upon the oxygen without the possibility of a corresponding reaction. But such mechanisms are at present unknown in physicochemical systems.

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However this may be, the investigation of Christiansen, Douglas, and Haldane (3) has proved that oxygen does influence the tension of carbon dioxide in blood in the manner indicated by the theory.

II.

In blood all the carbonic acid is present either as free acid ($\text{CO}_2 + \text{H}_2\text{CO}_3$) or as bicarbonate ($\text{HCO}_3 + \text{BHCO}_3$). This conclusion, originally reached from a study of the general acid-base equilibrium of the blood (4, 5), has been verified with great accuracy by the investigation of Hasselbalch (6). If we express the concentration of free carbonic acid by $[\text{CO}_2]$ and the concentration of total bicarbonate including both undissociated molecules and the bicarbonate ions by $[\text{BHCO}_3]$, we may write the expression for the equilibrium between these substances in the approximate form

$$k_{\text{CO}_2} = [\text{H}]^+ \frac{[\text{BHCO}_3]}{[\text{CO}_2]} \quad (5)$$

Here k_{CO_2} is a constant having for blood a value appreciably greater than that of the ionization constant of carbonic acid.

Equations 4 and 5 define the conditions for equilibrium between the hydrogen ion, carbonic acid, oxygen, hemoglobin, and bicarbonates in human blood. They are known to be true, at least with a fair approach to accuracy, on purely experimental grounds, independently of all theoretical considerations.

Several important conclusions follow directly from Equations 4 and 5. All of these, however, may be more clearly understood with the help of further theoretical considerations.

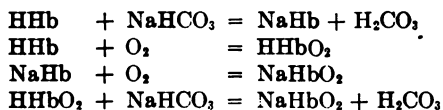
III.

The influence of carbonic acid upon the equilibrium between oxygen and hemoglobin must depend upon a chemical reaction in which both hemoglobin and carbonic acid are involved. Let us first consider the direct combination whereby hemoglobin bicarbonate is formed. For this purpose we may use Bohr's investigation (7) of the absorption of carbon dioxide by purified hemoglobin solutions.

From his numerous measurements Bohr drew the conclusion that the amount of carbonic acid united with hemoglobin varies with the tension of carbon dioxide so that, within those ranges of tension which exist in the blood, it amounts to about 0.5 cc. of CO_2 per gm. of hemoglobin. Bohr's experiments, however, were carried out in the absence of inorganic bicarbonates. Accordingly, the hydrogen ion concentration must have been very much higher than in blood. It follows that the amount of carbonic acid united to hemoglobin in blood must be at most very small, probably not more than 1 or 2 per cent by volume, and the variation of this quantity must be practically negligible.³ This subject will be discussed below.

Moreover, as Bohr showed (8), the union of carbonic acid with hemoglobin is uninfluenced by the presence of oxygen, so that this process cannot be involved in the phenomenon with which we are now concerned. In fact, as Bohr pointed out (8), it must be the globin portion of the hemoglobin molecule which combines with carbonic acid.

Secondly, we may examine the problem of the distribution of a base between carbonic acid and hemoglobin functioning as an acid. This seems to be the only remaining possibility of explaining the interaction of oxygen and carbon dioxide. It follows from this consideration, as suggested by Hasselbalch and Lunds-gaard (9), and by Christiansen, Douglas, and Haldane (3), and then more strongly asserted by Hasselbalch (6), and by Parsons (10), that oxyhemoglobin must be a stronger acid than reduced hemoglobin. Of course it also follows that the salts of hemoglobin must have a greater affinity for oxygen than has acid hemoglobin itself. Accordingly we seem to be concerned with the following different reactions:



Moreover, all the substances involved in these reactions, except oxygen, ionize in a greater or less degree. In order to study this

³ I discussed this question with Professor Bohr at the Heidelberg Congress in 1907.

question we may turn to the data of Christiansen, Douglas, and Haldane (3), from which the first four columns of Table I are constructed by means of the curves on page 256 of their paper. Column 1 gives carbon dioxide pressures in mm.; Column 2 the corresponding concentrations of free carbonic acid in volumes per cent; Column 3 the total carbonic acid absorption of the blood when equilibrium is established in the presence of oxygen, so that practically all the hemoglobin is in the form of oxyhemoglobin; Column 4 the corresponding value when oxygen is absent, so that all the hemoglobin is in the reduced condition. From these values those of Column 5 = Column 3 - Column 2 and

TABLE I.

(1) CO ₂	(2) [CO ₂]	(3) Total CO ₂ O.*	(4) R.*	(5) [BHCO ₂] O.	(6) R.	$\frac{+}{[H]} \times 10^9 N$	
						(7) O.	(8) R.
mm.	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent		
5	0.34	20.0	24.0	19.7	23.7	1.28	1.09
10	0.67	28.5	32.5	27.8	31.8	1.86	1.67
20	1.34	38.5	43.5	37.2	42.2	2.92	2.61
30	2.02	46.5	51.5	44.5	49.5	3.78	3.43
40	2.69	51.5	57.0	48.8	54.3	4.62	4.30
50	3.36	56.0	62.0	52.6	58.6	5.44	4.95
60	4.03	59.5	66.0	55.5	62.0	6.20	5.65
70	4.70	63.0	69.5	58.3	64.8	6.93	6.30
80	5.38	66.5	73.0	61.1	67.6	7.63	6.97

* O = oxidized blood; R = reduced blood.

Column 6 = Column 4 - Column 2 are calculated. These give the combined carbonic acid of the fully oxygenated and of the fully reduced blood. Columns 7 and 8 give the corresponding values of hydrogen ion concentration in accordance with Equation 5 and the research of Hasselbalch (6). Column 7 refers to the oxidized and Column 8 to the reduced blood.

In Fig. 2 the values of Columns 5 and 6 are plotted against those of Columns 7 and 8, thus illustrating the change in combined carbonic acid which accompanies change in hydrogen ion concentration in fully oxidized and fully reduced blood, respectively.

Points on these two curves having the same abscissa are isohydric points. Accordingly the difference of their ordinates represents the amount of base transferred from carbonic acid to hemoglobin when fully reduced blood is fully saturated with

Vol. per cent

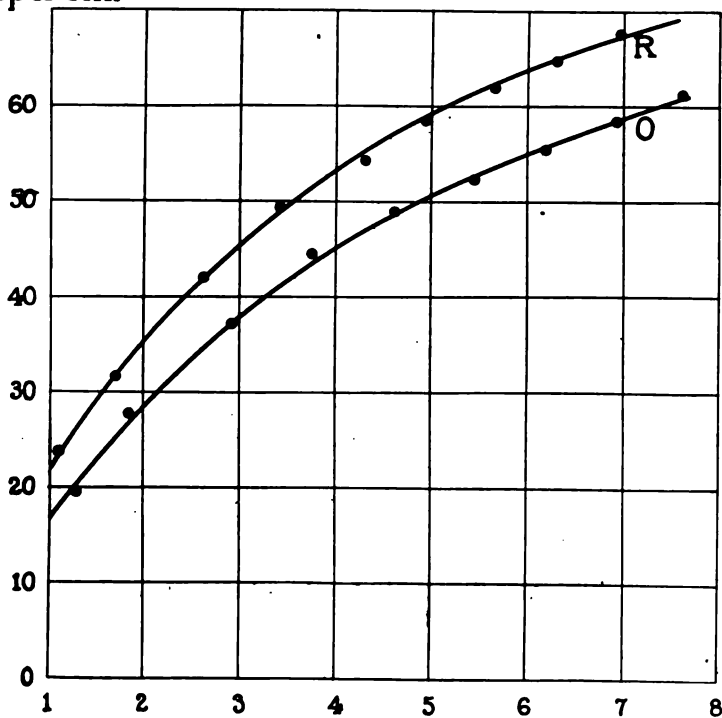


FIG. 2. Combined CO_2 as a function of $[\text{H}]^+$. Upper curve fully reduced blood; lower curve fully oxygenated blood of J. S. H. Ordinates are combined CO_2 in volumes per cent; abscissæ $[\text{H}] \times 10^9 \text{N}^+$.

oxygen isohydrically; *i.e.*, in such a manner, by regulating the tension of carbon dioxide, as to keep the hydrogen ion concentration constant. The only acids that can be involved in this reaction are hemoglobin and carbonic acid, because while the hydrogen ion concentration remains constant other acid substances can neither combine with nor liberate base. But car-

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bonic acid can do this if the tension of carbon dioxide is varied, and hemoglobin can do it if, by combining with oxygen, it becomes a stronger acid. Fig. 2 therefore affords a rigorous proof of the variation of the affinity of hemoglobin for base with variation in the amount of oxygen combined with hemoglobin. Thus the study of the isohydric properties of the system enables us to draw conclusions regarding the acid nature of hemoglobin. For the acid radical of hemoglobin which increases in strength when oxyhemoglobin is formed we may write mass law equations analogous to Equation 5 for carbonic acid.

$$k_R = \frac{+}{[H]} \frac{[BHb]}{[HHb]} \quad (6)$$

$$k_O = \frac{+}{[H]} \frac{[BHbO_2]}{[HHbO_2]} \quad (7)$$

It will be convenient to express concentrations of the various hemoglobin compounds in units of volumes per cent of oxygen and CO₂, noting that [BHb] and [BHbO₂] stand for the total concentrations, both ionized and non-ionized, of the two salts. If we assume that the total concentration of hemoglobin for the blood of J. S. H. corresponds to 18 per cent by volume of oxygen or carbon dioxide and write

$$\frac{[BHbO_2] - [BHb]}{[BHb]} = \frac{[BHCO_2]_R - [BHCO_2]_O}{[BHb]} = \Delta s$$

we find

$$\begin{aligned} [HHb] &= 18 - s \\ [BHbO_2] &= s + \Delta s \\ [HHbO_2] &= 18 - s - \Delta s \end{aligned}$$

values which hold for isohydric solutions both inclusively and exclusively.

The mass law equations then become

$$k_R = \frac{+}{[H]} \frac{s}{18 - s} \quad (8)$$

$$k_O = \frac{+}{[H]} \frac{s + \Delta s}{18 - s - \Delta s} \quad (9)$$

In order to solve these equations it is necessary to obtain two sets of values for $^+[\text{H}]$ and Δs . These may be obtained from Fig. 2, for example,

$$\begin{aligned} ^+[\text{H}]_1 &= 2 \times 10^{-8} & \Delta s_1 &= 6.7 \\ ^+[\text{H}]_2 &= 6 \times 10^{-8} & \Delta s_2 &= 8.8 \end{aligned}$$

whence

$$\begin{aligned} s_1 &= \frac{27 s_2}{9 + s_2} \\ \frac{s_1 + 6.7}{11.3 - s_1} &= \frac{3 s_2 + 26.4}{9.2 - s_2} \end{aligned}$$

and

$$\begin{aligned} s_1 &= 9.64 \\ s_2 &= 5.00 \end{aligned}$$

Using these values we find for the two cases in question the values of Table II.

TABLE II.

$^+[\text{H}]_1 = 2 \times 10^{-8}$	$[\text{BHb}]_1 = 9.64$	$[\text{BHbO}_2]_1 = 16.34$	$k_R = 2.3 \times 10^{-8}$
	$[\text{HHb}]_1 = 8.36$	$[\text{HHbO}_2]_1 = 1.66$	$k_O = 19.7 \times 10^{-8}$
$^+[\text{H}]_2 = 6 \times 10^{-8}$	$[\text{BHb}]_2 = 5.00$	$[\text{BHbO}_2]_2 = 13.80$	$k_R = 2.3 \times 10^{-8}$
	$[\text{HHb}]_2 = 13.00$	$[\text{HHbO}_2]_2 = 4.20$	$k_O = 19.7 \times 10^{-8}$

From these values of k_R and k_O and Equations 8 and 9 it is possible to calculate all isohydric values of $[\text{BHb}]$ and $[\text{BHbO}_2]$, and therefore of Δs . Some of these are given in Table III.

TABLE III.

(1) $^+[\text{H}] \times 10^8 \text{N}$	(2) $[\text{BHb}]$ <i>vol. per cent</i>	(3) $[\text{BHbO}_2]$ <i>vol. per cent</i>	(4) $\Delta s = (3) - (2)$ <i>vol. per cent</i>
1	12.5	17.1	4.6
2	9.6	16.3	6.7
3	7.8	15.6	7.8
4	6.6	15.0	8.4
5	5.6	14.4	8.8
6	5.0	13.8	8.8
7	4.5	13.3	8.8
8	4.0	12.8	8.8
9	3.7	12.4	8.7

It may readily be seen that these values of Δs approximately correspond with the differences in the ordinates of the two curves of Fig. 2. Only in the neighborhood of $[H] = 1 \times 10^{-8}$ is there a significant discrepancy. But since the shape of the curves of Christiansen, Douglas, and Haldane is uncertain in this range ($[CO_2] < 10$ mm.) it is impossible to attach any significance to this difference. On the other hand it must be carefully noted that the relative values of k_R and k_O depend upon differences between the several values of Δs obtained from the data of Christiansen, Douglas, and Haldane. These differences are highly uncertain. Accordingly it is hardly worth while to attempt accurately to determine the absolute magnitude of the constants. Nevertheless it is easy to show mathematically that the range of this variation consistent with the general character of the data of Christiansen, Douglas, and Haldane is very limited. Accordingly, we may say that the order of magnitude of these quantities is given by the equations

$$k_R = 2.3 \times 10^{-8} \quad (10)$$

$$k_O = 2.0 \times 10^{-7} \quad (11)$$

IV.

While the considerations above set forth afford a possible, and, as I believe, the real explanation of the influence of oxygen upon the absorption of carbon dioxide by blood, it is evident that the fluctuation in the amount of alkali combined with a single acid radical of hemoglobin is far from sufficient to account for the variation of $[BHCO_3]$ with varying $[H]$. The facts are illustrated in Table IV. Column 4 gives the quantities of base that can be accounted for, on the above considerations, in the blood at the hydrogen ion concentrations indicated in Column 1. It will be seen that for $[H] = 7 \times 10^{-8}N$ basic radicals chemically equivalent to about 70 per cent by volume of carbon dioxide are in evidence, while at a hydrogen ion concentration one-fourth as great an amount equivalent to only 40 per cent by volume is accounted for. What has become of the remainder? In some measure basic radicals have become free, as in the case of a portion of those of globin which were directly united with carbonic acid.

This is, however, a negligibly small factor. The great majority of the missing basic radicals have combined with other weak acid radicals. Thus a large fraction of whatever phosphoric acid may be present has been converted from molecules of the type BH_2PO_4 to B_2HPO_4 . But this again represents a small fraction of the total.⁴ Probably a considerable number of other substances are more or less involved in this same manner. But in a considerable degree it must be the acid radicals of the proteins which are in question. Of these there is an indefinitely large supply. They are present in the protein portion of the hemoglobin molecule and in all the proteins of the plasma. In short we come back to the old reaction, so often discussed,

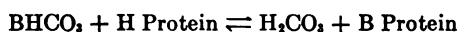


TABLE IV.

(1) $\frac{+}{[\text{H}]} \times 10^{\circ}\text{N}$	(2) [BHCO ₃] ₀	(3) [BHbO ₃]	(4) B = (2) + (3)
	<i>vol. per cent</i>	<i>vol. per cent</i>	<i>vol. per cent</i>
2	29.6	16.3	46
3	38.0	15.6	54
4	44.8	15.0	60
5	50.3	14.4	65
6	54.9	13.8	69
7	58.5	13.3	72

It is important to consider this reaction because there is danger of confounding the unique case of hemoglobin above discussed with the universal property of all proteins and so overlooking or misconceiving the latter.⁵ Moreover the acid radical of hemoglobin which has been in question is not only unique in its properties, it is probably not even a protein radical at all, but a radical of the hematin part of the molecule.

⁴ My original discussion of the theory of neutrality regulation (4, 5) has often been misunderstood at this point. I have not intended to imply that phosphates are important in the regulation of the reaction of the blood, unless in blood cells of species where much phosphoric acid is present, but rather in the body as a whole, *i.e.* in the tissues, and in the process of excretion.

⁵ This appears to be the only source of difference between certain conclusions which Parsons has reached (10) and the corresponding conclusions of this paper.

Every protein molecule contains a considerable number of acid and basic radicals. The researches of Sørensen (12) show that these radicals obey the ordinary laws of chemical equilibrium and my own experience with gluten (13) indicates that they conform to the simple laws with considerable accuracy, even under the most varied colloidal conditions. But since the several acid radicals of a protein possess different ionization constants the behavior of a protein, as the hydrogen ion concentration varies, corresponds, not to that of the solution of a single weak acid, but to that of a mixture of a considerable number of weak acids. Thus, if we calculate an ionization constant in the usual manner, we find that this varies with the hydrogen ion concentration. Nevertheless the change in this apparent ionization constant for small changes of hydrogen ion concentration is small and it ought not to be difficult roughly to test the question whether the protein may probably be accounted the cause of a considerable part of the variation in $[\text{BHCO}_2] + [\text{BHbO}_2]$ with varying $[\text{H}^+]$.

From Table IV it may be seen that on the assumption that proteins alone are involved, the variation in the amount of base in union with protein in blood between $[\text{H}^+] = 1 \times 10^{-8}\text{N}$ and $[\text{H}^+] = 1 \times 10^{-7}\text{N}$ must be equivalent to about 50 per cent by volume of carbonic acid. Such a variation of the quantity of base would correspond, in round numbers, to 2 mols of base per mol of protein in the blood as a whole, assuming that the other proteins average the same molecular weight as hemoglobin. In other words, while passing from $[\text{H}^+] = 1 \times 10^{-8}\text{N}$ to $[\text{H}^+] = 1 \times 10^{-7}\text{N}$, somewhat less than 10,000 gm. of the mixed protein of the whole blood must give up 1 mol of base, if the phenomenon is ascribed to the basic radicals of the proteins alone.

It is easy to see that if, on the average, every 500 gm. of blood protein contains 1 mol of acid radical of such a nature that in the range between $[\text{H}^+] = 1 \times 10^{-8}\text{N}$ and $[\text{H}^+] = 1 \times 10^{-7}\text{N}$ all these radicals together behaved approximately like so many acid radicals of the strength $k = 5 \times 10^{-10}$ the necessary condition would be fulfilled. This appears to be a possible assumption, although it is not impossible that the proteins are on the whole even weaker acids and that small quantities of other substances

such as weak organic acids or organic compounds of phosphoric acid may be involved in a small degree. For comparison it may be noted (13) that about 30,000 gm. of gluten are required to liberate 1 mol of base between $[\text{H}^+] = 1 \times 10^{-8}\text{N}$ and $[\text{H}^+] = 1 \times 10^{-7}\text{N}$. In the case of the serum proteins (14, 15) the necessary amount of protein is only about 15,000 gm. It must be remembered, however, that globin differs from these.

Certain observations of Hasselbalch,⁶ although inconclusive, suggest that the acid radicals of globin may be strong enough to account for nearly the whole change within the corpuscles. The isoelectric point of hemoglobin suggests the same conclusion.

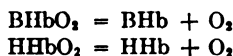
In any case it is evident that the facts are consistent with the general theory of acid-base equilibrium and the well established properties of proteins. The newly discovered property of hemoglobin does not lead to new difficulties, but, on the contrary, removes at least one serious difficulty of long standing. It is to illustrate this fact that the present section has been written, and no claim is made whatever for the accuracy of the estimates which are involved.

V.

Equations 6 and 7 yield the equation

$$\frac{k_O}{k_R} = \frac{[\text{BHbO}_2] \cdot [\text{HHb}]}{[\text{BHb}] \cdot [\text{HHbO}_2]} \quad (12)$$

For infinite dilution the mass law equations for the reactions



have the simple form

$$k_s = [\text{O}_2] \frac{[\text{BHb}]}{[\text{BHbO}_2]} \quad (13)$$

$$k_A = [\text{O}_2] \frac{[\text{HHb}]}{[\text{HHbO}_2]} \quad (14)$$

whence

$$\frac{k_A}{k_s} = \frac{[\text{BHbO}_2] \cdot [\text{HHb}]}{[\text{BHb}] \cdot [\text{HHbO}_2]} \quad (15)$$

⁶ Hasselbalch (6), p. 130.

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and from Equations 12 and 15

$$\frac{k_A}{k_s} = \frac{k_O}{k_R} \quad (16)$$

Finally this expression, together with the approximations of Equations 10 and 11, gives

$$\frac{k_A}{k_s} = 9 \quad (17)$$

In spite of the fact that Equation 17 is a very rough approximation, it will be convenient to employ it as a means of estimating the probable effect that carbonic acid exerts upon the

TABLE V.

(1) + [H] × 10 ⁶ N	(2) [BHb]	(3) [HHb]	(4) k_1 Calculated.	(5) K Barcroft.	(6) $\frac{(5)}{(4)}$
	<i>vol. per cent</i>	<i>vol. per cent</i>			
1	12.5	5.5	3.6	764	210
2	9.6	8.4	5.1	1,265	250
3	7.8	10.2	6.0	1,980	330
4	6.6	11.4	6.6	2,770	420
5	5.6	12.4	7.0	3,620	520
6	5.0	13.0	7.4	4,560	620
7	4.5	13.5	7.7	5,560	720
8	4.0	14.0	7.9	6,700	850
9	3.7	14.3	8.1	8,000	990

affinity of hemoglobin for oxygen. In Table V, Columns 1 and 2 of Table III are reproduced, Column 3 gives values of [HHb] = 18 - [BHb], Column 4 gives values of the apparent constant $k = [\text{O}_2] \frac{[\text{Hb}]}{[\text{HbO}_2]}$ which these imply, according to the relation⁷

$$k = k_s \frac{[\text{BHb}]}{18} + 9 k_A \frac{[\text{HHb}]}{18}$$

These calculated values of k correspond to the requirements of the theory for fully reduced blood at infinite dilution. The differences in the values are maxima, for as the blood takes up

⁷ Note that $9k_s = k_A$.

oxygen there is increasingly less and less difference between the amounts of base in union with hemoglobin at any two hydrogen ion concentrations. Column 5 contains values of k (equal to $\frac{1}{K}$ of Hill's formula) obtained from Barcroft's data. The scales of the two k 's differ and it is therefore necessary to compare their ratios, which are given in Column 6.

Evidently there is no agreement between k as calculated and the K of Barcroft and Hill, save that they vary in the same sense. But this is inevitable, since the original assumptions, on which the value of the calculated k depends, rest upon the phenomenon which finds expression in Hill's formula in an increase of k , i.e., a decrease of Hill's K , with increase of $[H]$; i.e., with increase of $[CO_2]$.

This is the first real difficulty which we have encountered. The difficulty is real, because, first, the value of $\frac{k_A}{k_s}$, though uncertain, seems to be far too small to account for the variation in Barcroft's K , and secondly because it is evident that Barcroft's K should vary in accordance with the variation of k as theoretically calculated. This may readily be seen by writing equations according to Hill for the dissociation of acid oxyhemoglobin and its salt.

$$k_A = \frac{[HHb] \cdot [O_2]^{3.5}}{[HHbO_2]}$$

$$k_s = \frac{[BHb] \cdot [O_2]^{3.5}}{[BHbO_2]}$$

Then, as before,

$$\frac{k_A}{k_s} = \frac{[BHbO_2] \cdot [HHb]}{[BHb] \cdot [HHbO_2]}$$

Therefore, if Hill's expression not only permits the accurate calculation of points on the oxygen dissociation curve of blood, but also represents the real mechanism of the reaction, it appears to follow that

$$\frac{k_A}{k_s} > 100$$

Indeed it would seem probable that this is far too low an estimate, because, throughout the greater part of the absorption curves, when oxygen has already caused a further union between hemoglobin and base the values of k may be expected to vary less and less between the curves of different tensions of carbon dioxide.

This consideration, moreover, casts a doubt upon the validity of Hill's curves as an expression of the real mechanism of the reaction. For, since the union of oxygen with hemoglobin must increase the union of hemoglobin with base and since, on general thermodynamical principles, this must in turn increase the affinity of hemoglobin for oxygen, it follows that, unless there is some compensatory process of an unknown nature, the value of k for a given tension of carbon dioxide must be itself a function of $[O_2]$ and therefore a variable. Thus it may be seen that the problem is somewhat more complex than is implied even by the searching analysis of Barcroft and Hill.

Returning to the discrepancy between the calculated values of k and those given by Barcroft and Hill, it may be said, on chemical grounds, that an increase in the affinity of the acid radical in question greater than that obtained by calculation, for example,

$$4 < \frac{k_A}{k_s} < 20$$

is hard to accept.

It seems necessary to suppose that this acid radical is located in the hematin portion of the molecule, and that its position is very close to that taken by oxygen when it combines with hemoglobin. If the position were α or β to that of an atom with which oxygen is united, a sensible effect upon the two affinities would correspond with general chemical experience. This is also the reason for assuming that a single acid radical is in question. It seems certain that this number must be very small. At a much greater distance analogy suggests that this effect is out of the question. Perhaps the difference of the ionization constants of propionic acid, $k = 0.000014$, and lactic acid, $k = 0.00014$, is worth consideration.⁸ It must not be forgotten, however, that the heat of formation of lactic acid from propionic acid is much greater than the heat of formation of oxyhemoglobin from hemoglobin.

⁸ This same comparison has been made by Parsons (10).

On the other hand it is to be considered that in the latter case 2 atoms of oxygen are involved. And of course it is not inconceivable, although probably it does not agree with conceptions now held, that the addition of oxygen takes place upon the very atom which binds the alkali also.

The data of Christiansen, Douglas, and Haldane seem to be inconsistent with the conceivable hypothesis that the dissociable oxygen is an essential part of this acid radical, for then the values of Δs would be much higher in the more alkaline than in the more acid range.

Finally, however, it seems almost certain that, if the difference between k_A and k_s were of the order of magnitude indicated by Hill's theory, every preparation of blood in which there is a considerable amount of oxygen in combination would have nearly the same affinity for base, and therefore for oxygen, as every other, regardless of the tension of carbon dioxide. If this is true, Hill's equation cannot be regarded as a valid expression of the real mechanism of the reaction, though I have no doubt of its validity as a means of calculating points on the absorption curves.

Stated in the most general terms, the work of Barcroft conclusively proves that, in the presence of electrolytes and therefore in blood, the active mass of hemoglobin is different from that value which can be calculated from the absorption of oxygen. If we take this conclusion for granted and abandon the attempt theoretically to account for it, just because we are thereby freed from a theoretical interpretation, we are able tentatively to take a further step. For it seems to be probable, and I think the constancy of Hill's n for all values of $[\text{CO}_2]$ strongly supports the view, that with a given percentage saturation of hemoglobin, regardless of the tension of carbon dioxide, the active masses of oxyhemoglobin and of reduced hemoglobin are more or less roughly constant. For example, it seems justifiable to assume that for all samples of blood which are just half saturated with oxygen approximately

$$\frac{[\text{Hb}]}{[\text{HbO}_2]} = \frac{[\text{HHb}] + [\text{NaHb}]}{[\text{HHbO}_2] + [\text{NaHbO}_2]} = \text{Constant}$$

though of course it is by no means equally possible to assume that

$$\frac{[\text{Hb}]}{[\text{HbO}_2]} = 1$$

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If this is true the expression

$$k = [\text{O}_2] \frac{[\text{Hb}]}{[\text{HbO}_2]}$$

may be assumed to hold, with the qualification that we have at present no means of estimating the values of $[\text{Hb}]$ or $[\text{HbO}_2]$, which now stand for the true active masses, or even of $\frac{[\text{Hb}]}{[\text{HbO}_2]}$.

Nevertheless there is reason to suppose that the values of this ratio are constant for all cases where the saturation of the blood with oxygen has the same value, regardless of the tension of carbon dioxide.

This enables us to use Barcroft's curves for the calculation of values of k which, for any given degree of saturation of the blood

TABLE VI.

$\frac{[\text{Hb}]}{[\text{HbO}_2]}$	9.00	2.33	1.00	0.43	0.11
$[\text{CO}_2]$	Oxygen tensions.				
mm.	mm.	mm.	mm.	mm.	mm.
0	4	7	10	15	26
3	6	10	14	20	35
20	8	14	20	29	49
40	10	18	26	37	61
90	14	25	35	50	85

with oxygen, *i.e.* for any given ordinate, are probably comparable, though for different ordinates they are not comparable.

Table VI gives data taken from Fig. 1 of the values of $[\text{O}_2]$ in mm.

These data of Table VI yield the relative values of k given in Table VII. The calculation consists in dividing the oxygen tension for any particular degree of saturation and carbon dioxide tension by that for the same percentage saturation and 0 mm. of carbon dioxide tension.

It must be understood that the values of k given by Table VII are relative, and comparable only within each column of the table taken by itself. It is evident, however, that aside from errors in reading Barcroft's curves all the columns are identical, so that Table VII reduces to the very simple form of Table VIII.

Of course the identity of the columns of Table VII depends upon a mathematical property of Hill's equation, and the exact agreement cannot therefore be significant for the present discussion. But since Barcroft's curves agree, within the limits of experimental accuracy, with the observations, we are nevertheless justified in using Table VIII, which has just as much validity as the assumption that for any one percentage saturation the active masses of hemoglobin and of oxyhemoglobin are constant with varying tensions of carbon dioxide. And of course it has no more validity than this.

TABLE VII.
Values of k .

1.00	1.00	1.00	1.00	1.00
1.50	1.43	1.40	1.33	1.35
2.00	2.00	2.00	1.93	1.89
2.50	2.55	2.60	2.47	2.35
3.50	3.55	3.50	3.33	3.27

TABLE VIII.

[CO ₂]	k
mm.	
0	1.0
3	1.4
20	2.0
40	2.5
90	3.4

We are now in a position to compare values of k obtained from Barcroft's data by an assumption which, I think, is certainly not less reasonable than that involved in Hill's equation, with values of k calculated from the values of k_A and k_s . For this purpose we may take the case of fully reduced blood as represented by Column 4 of Table V. Table IX gives the necessary values.

Column 4 of Table IX, giving the ratios of the two values of k , unlike the similar column of Table V, contains values which are roughly constant. I think we are therefore justified in saying that, after all, the influence of carbonic acid upon the affinity of blood for oxygen does probably correspond with the requirements

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of the theory of acid-base equilibrium. At any rate there is certainly no decisive evidence of inconsistency.

Since Barcroft has proved the active masses of hemoglobin and oxyhemoglobin to be different from the apparent values, it is evident that there may be a discrepancy between the combination of hemoglobin with base as indicated by theory, and the actual facts. This consideration raises a doubt regarding the validity of the present quantitative conclusions in general, which the above calculation cannot wholly remove. It must be remembered, however, that there is much evidence to prove that under all sorts of colloidal conditions the union of proteins with bases and acids corresponds closely with the requirements of the mass law. We are therefore not only permitted but obliged to go as far with this theory as we can.

TABLE IX.

(1) [CO ₂]	(2) k Table VIII.	(3) k Calculated.	(4) $\frac{(3)}{(2)}$
<i>mm.</i>			
3	1.4	3.8	2.7
20	2.0	5.7	2.9
40	2.5	6.7	2.7
90	3.4	7.8	2.3

Moreover, if we write equations as follows:

$$k_R = [\text{H}]^{2.5} \frac{[\text{BHb}]}{[\text{HHb}]}$$

and

$$k_O = [\text{H}]^{2.5} \frac{[\text{BHbO}_2]}{[\text{HHbO}_2]} .$$

which correspond to Hill's assumption, it appears that the values

$$k_R = 7.8 \times 10^{-20}$$

$$k_O = 110 \times 10^{-20}$$

yield results which agree roughly with the facts and permit the same conclusions that have been deduced from the assumptions made in this paper. It will be seen that even here the value of

the ratio $\frac{k_O}{k_R}$ corresponds with what is to be expected on chemical grounds.

It remains true, however, that we have no satisfactory means of accounting for the fact that according to Hill's formula, at least in its original implication, the difference in affinity for oxygen at any two tensions of carbon dioxide is independent of the amount of combined oxygen. The doubts expressed by Barcroft,⁹ concerning the validity of the theoretical curves in their upper ranges, nevertheless suggest a possible escape from this final complication.

VI.

Bohr's data (11) of the absorption of carbon dioxide by solutions of hemoglobin make possible a rough estimate of the extent to which hemoglobin combines with carbonic acid in blood. For example, we may make use of his results for a solution containing 3.8 per cent hemoglobin.

In Table X, Column 1 gives the tension of carbonic acid, Column 2 the dissolved free carbonic acid, Column 3 total dissolved carbonic acid, and Column 4 (Column 3—Column 2) combined carbonic acid in cc. of gas for 37.8 cc. of solution. From these data Column 5 is calculated according to the expression

$$[H]^+ = 6 \times 10^{-7} \frac{[CO_2]}{[BHCO_3]}$$

The values of Column 5 represent minimum values for the hydrogen ion concentration, which are probably sensibly too low and which must therefore lead to an overestimate rather than to an underestimate of the combination of hemoglobin with carbonic acid in blood.

Extrapolation indicates that for $[H]^+ = 0.35 \times 10^{-7}N$ the value of $[BHCO_3]$ should be about 0.75 cc., which agrees with the approximation that for low values of the hydrogen ion concentration, the value of $[BHCO_3]$ should be directly proportional to $[H]^+$. This quantity is an estimate of the total quantity of acid which may be regarded as combined with hemoglobin functioning as a base at $[H]^+ = 0.35 \times 10^{-7}N$ in 37.8 cc. of solution containing

⁹ Barcroft (1), p. 59.

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3.8 per cent hemoglobin. From this the total salt formation s of hemoglobin functioning as a base, expressed in units of per cent by volume, for blood containing 14 per cent hemoglobin may be obtained as follows:

$$s = \frac{0.75}{37.8} \times \frac{14}{3.8} \times 100$$

whence

$$s = 7.3 \text{ per cent by volume}$$

This estimate represents the sum of the concentrations of all undissociated salt molecules formed of hemoglobin with all the

TABLE X.

(1) CO ₂	(2) [CO ₂]	(3) Absorbed CO ₂	(4) [BHCO ₂]	(5) $\frac{+}{[H]} \times 10^7 N$
mm.	cc.	cc.	cc.	
6.04	0.275	2.098	1.823	0.90
11.57	0.527	2.885	2.358	1.35
14.62	0.666	3.230	2.564	1.55
18.54	0.844	3.666	2.822	1.80
24.07	1.095	4.197	3.102	2.10
31.98	1.455	4.855	3.400	2.55
43.14	1.963	5.715	3.752	3.15
60.03	2.731	6.815	4.084	4.05
85.40	3.886	8.335	4.449	5.25
124.96	5.684	10.507	4.823	7.10
188.62	8.583	13.828	5.245	9.85

acids of the corpuscles, plus the concentration of hemoglobin cations. Since these salts include those of all acids, it seems safe to say that hemoglobin bicarbonate cannot amount to even one-third of the total. Roughly about 2 per cent by volume seems therefore to be the highest possible estimate. It is also evident that for changes of hydrogen ion concentration, which are physiologically significant, the change in concentration of hemoglobin bicarbonate is negligible.

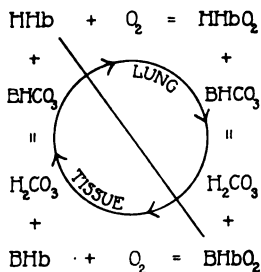
Experiments with blood involving wider changes of hydrogen ion concentration may, however, lead to sensible variations in the concentration of hemoglobin bicarbonate. Thus, for the range $\frac{+}{[H]} = 1 \times 10^{-8} N$ to $\frac{+}{[H]} = 1 \times 10^{-7} N$ the variation of hemo-

globin bicarbonate concentration is perhaps nearly 5 per cent by volume, and, as Table X shows, for higher values of $[H]^+$ this variation may be important. It must be noted, however, that such ranges of reaction cannot be reached except in the absence of the normal bicarbonates of the blood and in the presence of carbon dioxide at high tensions.

VII.

The changes which actually take place in the blood as a result of absorption of oxygen and escape of carbonic acid in the lung, followed by escape of oxygen and absorption of carbonic acid in the tissue, may now be considered. It is true that for this purpose we do not yet possess all the desirable experimental information, and it is evident that quantitatively the considerations developed in the preceding sections leave much to be desired. Nevertheless there seems to be little doubt about the general characteristics of the physiological process.

In the lung the escape of carbonic acid causes a transfer of base from this acid to the proteins, the phosphates, etc., and to the acid radical peculiar to hemoglobin. Meanwhile the absorption of oxygen leads to an increase in the concentration of oxyhemoglobin. Each of these processes facilitates the other, for while union of base with hemoglobin increases the affinity for oxygen the union of oxygen with hemoglobin increases the affinity for base. In the tissues these processes, and with them the affinity changes, are reversed. Such is the nature of the primary phenomenon, in accordance with the theoretical considerations of Sections III and V of this paper. An incomplete representation of the process is given by the accompanying diagram:



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Certain cases of this cycle, which are indeed beyond the range actually occurring in the body, but which illustrate the principles involved, are defined by the data and the deductions above set forth.

Let us consider, for example, the case of blood in equilibrium, at a temperature of 38° , with an atmosphere consisting of ordinary air to which enough carbon dioxide has been added to make its tension 20 mm. This blood, as we have seen, will have approximately the following composition:

CO ₂	Oxygen absorption.	[BHbO ₂]	[HHbO ₂]	[BHCO ₂]	[H] ⁺
mm.	per cent	vol. per cent	vol. per cent	vol. per cent	
20	100	15.7	2.3	37.2	$2.92 \times 10^{-8}N$

When this preparation is brought into equilibrium with an atmosphere free from oxygen in which the tension of carbon dioxide is 60 mm. the following conditions will be established:

CO ₂	Oxygen absorption.	[BHb]	[HHb]	[BHCO ₂]	[H] ⁺
mm.	per cent	vol. per cent	vol. per cent	vol. per cent	
60	0	5.3	12.8	62.0	$5.65 \times 10^{-8}N$

In such a case the effect of carbon dioxide upon the oxygen absorption is of course obscured. But, in default of other data, it is necessary to take instances where the oxygen absorption is either 0 or 100 per cent. Even here the *process* has, of course, been modified by the changing affinity of hemoglobin for oxygen. The defect is, however, not a serious one, since Barcroft's data (1), and those of Bohr, Hasselbalch, and Krogh (2) before him, sufficiently illustrate this point.

The increase in combined carbonic acid in the above imaginary experiment is evidently $62.0 - 37.2 = 24.8$ per cent by volume. This has been made possible by the liberation of an amount of base equivalent to $15.7 - 5.3 = 10.5$ per cent by volume from the acid radical peculiar to hemoglobin. The remainder of the base, equivalent to $24.8 - 10.5 = 14.3$ per cent by volume, has come from the proteins, including globin, and other buffer substances. This estimate is subject to a slight, but practically negligible, correction owing to the variation in the amount of hemoglobin bicarbonate.

If the hemoglobin did not vary in affinity for base, the increase in the amount of absorbed carbonic acid accompanying a change of hydrogen ion concentration from $2.92 \times 10^{-8}\text{N}$ to $5.65 \times 10^{-8}\text{N}$ would accordingly be only $\frac{14.3}{24.8} = 58$ per cent or roughly a little more than half the real change.

Again, if the hemoglobin did not vary in affinity for base the increase of the hydrogen ion concentration accompanying an increase of 24.8 per cent by volume in the combined carbonic acid would be more than 170 per cent of the real change. In other words the hydrogen ion concentration would have risen to more than $7.5 \times 10^{-8}\text{N}$.

Let us take two more conditions of equilibrium:

O ₂	CO ₂	Oxygen absorption.	[BHb]	[HHb]	[BHbO ₂]	[HHbO ₂]	[BHCO ₂]	⁺ [H]
mm.	mm.	per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	
0	40	0	6.3	11.7			54.3	$4.30 \times 10^{-8}\text{N}$
140	40	100			14.6	3.4	48.8	$4.62 \times 10^{-8}\text{N}$

From these data we find that when oxygenated blood with the tension of carbon dioxide of 20 mm. is converted into reduced blood with a carbon dioxide tension of 40 mm. the hydrogen ion concentration changes from $2.92 \times 10^{-8}\text{N}$ to $4.30 \times 10^{-8}\text{N}$. At the same time the carbonic acid increases by an amount equal to $54.3 - 37.2 = 17.1$ per cent by volume. The base yielded by the hemoglobin radical is $15.7 - 6.3 = 9.4$, and that yielded by ordinary buffer action, accordingly, $17.1 - 9.4 = 7.7$ per cent by volume.

Again, passing from oxygenated blood with a tension of carbon dioxide of 40 mm. to reduced blood with a tension of carbon dioxide of 60 mm. involves a change of hydrogen ion concentration from $4.62 \times 10^{-8}\text{N}$ to $5.65 \times 10^{-8}\text{N}$; the increase of combined carbonic acid is $62.0 - 48.8 = 13.2$ per cent by volume; the base yielded by the hemoglobin radical is $14.6 - 5.2 = 9.4$ per cent by volume, and that yielded by ordinary buffer action only $13.2 - 9.4 = 3.8$ per cent by volume. In this last case, if the peculiar function of the hemoglobin were eliminated, the change in absorption of carbonic acid for the given change of hydrogen ion concentration would be only $\frac{3.8}{13.2} = 29$ per cent of its real value.

The change in the hydrogen ion concentration for the given change in combined carbonic acid, however, would be at least 350 per cent of the real value and the concentration of the hydrogen ion would rise to more than $8 \times 10^{-8}N$.

Since data defining all the conditions for tensions of oxygen corresponding to the physiological range are not at hand, this question can hardly be further analyzed.

From the above data, however, it is evident that the type of change which involves a very great absorption of carbon dioxide for a very small change in concentration of the hydrogen ion and, of course, a very small change in hydrogen ion concentration for a very large change in carbonic acid absorption is particularly marked for tensions of carbonic acid which are physiologically important. Moreover the shape of Barcroft's curves affords at least equally good evidence that it is also predominant within ranges of oxygen tension corresponding to those which exist in arterial and venous blood.

If this phenomenon is of the first importance as a means of increasing the efficiency with which oxygen and carbonic acid are carried to and from the tissues, it appears to be no less important as a factor in regulating the hydrogen ion concentration of the blood. This has been pointed out by Christiansen, Douglas, and Haldane (3), by Hasselbalch (6), and others.

The possibility of such a process as this, depending upon a cyclic variation in the ionization constant of an acid was completely, though I hope pardonably, overlooked in my original description of the acid-base equilibrium of the organism (4). And a claim that all the different types of physicochemical phenomena which are involved in the process had been at least considered (5) must accordingly now be withdrawn. I think there can be little doubt, however, that this is the only example of such a chemical mechanism which has ever come to light.

This leads to a further physiological consideration. It has long been an open secret that it was impossible theoretically to account for the quantity of carbonic acid which escapes from the blood in the lung. The paper of Christiansen, Douglas, and Haldane went far to overcome the difficulty, but the fact of the very slight variation of hydrogen ion concentration, which was the kernel of the whole question, remained or at least was insufficiently ex-

plained. In the light of the present considerations I believe that the difficulty probably disappears.

One of the most puzzling phenomena attending changes of the oxygen and carbon dioxide content of the blood is the increase in the volume of the red cells, accompanied by a passage of acid radicals from the plasma into the corpuscles, when carbonic acid tension increases. The process is of course reversed by a decrease of carbon dioxide tension. Some years ago Spiro and I succeeded in producing somewhat similar phenomena in much simpler systems (16). As a result of these experiments we reached the conclusion that the process depends, in the main, upon the fact that as carbonic acid concentration increases there is a shift of base from proteins to carbonic acid. If this explanation is true, it is natural to suppose that more base will be given up within the corpuscles than in the plasma, since the concentration of proteins in the corpuscles is much greater. As a result the osmotic pressure will increase more, and the hydrogen ion concentration less, in the corpuscles than in the plasma; water will pass into the corpuscles to reestablish the osmotic equilibrium, and acid to reestablish the acid-base equilibrium between the cells and the solution in which they are suspended.

Evidently this process helps to explain the escape of carbonic acid from the plasma without a change of the hydrogen ion concentration of the magnitude that would otherwise be necessary. In this way it was possible to show that the plasma proteins have no great share in controlling the reaction of the blood (17). But this consideration, while valid, obviates one difficulty only to raise another. This new difficulty, however, concerns the affinity of hemoglobin for base. But, in view of the basic character of the protein globin, I was at that time disposed to think that it was hard to account for so great a fluctuation in the amount of base combined with hemoglobin as was clearly indicated by even the roughest estimates of the magnitude of the process. Variation in the affinity of hemoglobin for base puts the whole question in a different light, and Hasselbalch and Warburg, who hold the same view of the general nature of the process, have pointed out that this goes far to explain the phenomenon (18). The theory developed in the present paper permits an estimate of the quantitative variations involved.

Let us turn once more to the change from blood saturated with air containing carbon dioxide at a tension of 40 mm. to the system in equilibrium with an atmosphere free from oxygen, in which the tension of carbon dioxide is 60 mm. There is involved an increase in combined carbonic acid amounting to 13.2 per cent by volume. Of this nearly three-quarters will come from the hypothetical hematin radical of variable affinity and, of the remaining one-fourth, the buffer action of globin and other substances within the corpuscles must account for much the greater share, leaving probably not more than 1 per cent by volume as the share of the buffer substances of the plasma. But, since the volume of the corpuscles is less than that of the plasma, the actual changes in concentration will be even more disparate.

For every molecule of bicarbonate thus added there must be a net increase of at least 0.5 molecule or ion in solution. Accordingly there must be a large increase in the osmotic pressure of the corpuscles and a very small increase in the osmotic pressure of the plasma. Similarly, there must be a relatively large increase in the hydrogen ion concentration of the plasma, or at least a tendency in this direction, and a very small increase in the hydrogen ion concentration of the corpuscles, because the increase in the hydrogen ion concentration is checked by the increase of bicarbonate according to the implications of the familiar equation

$$[H]^+ = k \frac{[CO_2]}{[BHCO_3]}$$

Taking these arguments into account it seems safe to say that the main features of the phenomenon are probably known.

In conclusion it needs to be said that the validity of the somewhat difficult, and no doubt quantitatively provisional, theoretical discussions of the present paper is not necessary for the validity of the conclusions. The influence of carbon dioxide on oxygen tension is a well established fact (1, 2). So is the influence of oxygen on carbon dioxide tension (3), and from these facts the influence upon the hydrogen ion concentration and the acid-base equilibrium follow necessarily (4, 5). Given these three postulates the other phenomena may be deduced. What is uncertain is the exact magnitude of these; their existence is, I believe, no longer open to question. It is important not to attach too much signif-

icance to estimates, necessarily provisional, of these magnitudes and I hope before long to be able to report results of a research, recently resumed after interruption by the war, which will permit a revision of the estimates of this paper.

SUMMARY.

This paper seeks to explain the interaction between oxygen and carbonic acid in blood by means of the theory of acid-base equilibrium.

The isohydric change from fully reduced to fully oxygenated blood; the transfer of base from carbonic acid to hemoglobin, which is the main feature of this process; and the change of strength as acid of a portion of the hemoglobin molecule, which is its cause, are discussed. It is shown that all these phenomena can be explained by the assumption that a certain acid radical of the hemoglobin molecule has, for reduced hemoglobin and oxyhemoglobin respectively, the following values:

$$k_R = 2.3 \times 10^{-8}$$

$$k_O = 2.0 \times 10^{-7}$$

From this consideration it follows that the salt of the acid radical in question must have a greater affinity for oxygen than the free acid. If the mass law constants of the reaction of salt and acid with oxygen were k_s and k_A respectively, it should be approximately true that

$$\frac{k_A}{k_s} = \frac{k_O}{k_R} = 9$$

In the light of these considerations the equilibrium between protein acid radicals and base in blood is examined.

The bearing of these considerations upon Hill's equation for the equilibrium between hemoglobin and oxygen is considered, but at this point the difficulties are only partially overcome.

The union of hemoglobin as a base with acids, and especially with carbonic acid in blood, is discussed.

Finally, the bearing of these considerations upon the physiological processes, both the homogenous reactions within the corpuscles and the plasma, and also the heterogenous exchanges between corpuscles and plasma, is investigated.

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FERMENTATION OF FRUCTOSE BY *LACTOBACILLUS* *PENTOACETICUS*, N. SP.*

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The fermentation of fructose by the pentose fermenters which have been called *Lactobacillus pentoaceticus*, n. sp., presents some very interesting and unique aspects; viz., the production of mannitol and the subsequent fermentation of this mannitol into acetic and lactic acids.

The production of mannitol by the fermentation of fructose has been observed by several investigators and has led these investigators to designate the fermenting organisms as "mannite bacteria;" i.e., mannitol-forming bacteria.

Strecker was perhaps the first to obtain mannitol by a fermentation process. He allowed a mixture of hydrolyzed cane sugar, cheese, sour milk, and calcium carbonate to undergo spontaneous fermentation for 2 or 3 months. From the fermented mass he isolated mannitol in considerable quantities; e.g., 1 pound of pure mannitol from the fermentation of 10 pounds of sugar. Dragendorff repeated the earlier work of Strecker and obtained a good yield of mannitol in his first fermentation, but a second experiment failed to show a trace of mannitol.

In the course of a discussion on the causes and conditions of lactic acid production Pasteur pointed out that besides lactic acid, other products, such as alcohol, butyric acid, and mannitol, were formed. He noted that great variations occurred in the amount of mannitol produced.

Marcano in the making of rum from the sugar-cane of the West Indies recognized mannitol as a product of the fermentation.

In all this early work no attempt was made to use pure cultures of bacteria or pure chemical substances.

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Gayon and Dubourg made a very careful study of the formation of mannitol in fermenting solutions. From a white wine they isolated an organism whose power of fermentation they tested on various sugars and related compounds. From fructose, mannitol was obtained in considerable quantity. Sucrose was readily fermented but gave no mannitol. On the other hand, when sucrose was first hydrolyzed into its two components glucose and fructose and then fermented, mannitol was formed. From this evidence Gayon and Dubourg concluded that sucrose was fermented directly by the bacteria and not inverted before fermentation, as is usually considered to be the case.

In an extensive study of the mannitic fermentation of wine Müller-Thurgau and Osterwalder isolated a number of organisms that produced mannitol from fructose. One of these, *Bacterium mannitopæum*, they report was able to ferment sucrose with the formation of mannitol. In two experiments crystals of mannitol were observed, but the amount formed was apparently too small to determine quantitatively as no separation of the crystals was made. Aside from these two experiments with *Bacterium mannitopæum*, no report has been found in the literature of mannitol formation directly from sucrose by means of a pure culture of bacteria.

Mannitol is frequently used as a source of carbon in the cultivation of bacteria. The ability of numerous organisms to break down mannitol is thus well established. The mannitol-forming bacteria as a group do not seem to possess this power. The organisms isolated and studied by Gayon and Dubourg, and by Müller-Thurgau and Osterwalder failed to grow on a medium in which fermentable sugars were replaced by mannitol.

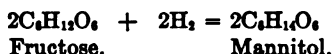
Laborde isolated three organisms from wine which seemed to have the power of forming mannitol from fructose and later destroying the mannitol. He analyzed the cultures at two successive times in the course of the fermentation and obtained less mannitol in the second analysis than he had found 2 months earlier. His paper gives no data concerning the products formed from the mannitol.

All the mannitol-forming bacteria studied by the above investigators have been isolated from various kinds of wine. The organisms which are described in this and in our preceding papers (1919, 1920) have been isolated from different sources, such as manure, silage, and soil, and have a more general distribution in nature than the so called "mannite bacteria." *They are related to these bacteria by their ability to form mannitol from fructose; but a more conspicuous and characteristic property is their power to bring about a very rapid and complete fermentation of the pentose sugars xylose and arabinose.* It is probable that the general occurrence of the pentose fermenters is closely correlated with the

equally wide distribution of pentose-yielding compounds and that these bacteria play an important part in the decomposition of these plant compounds.

As will be shown later in this paper, the pentose fermenters produce mannitol in such quantities that it can be easily isolated, identified, and subsequently fermented by the organism that produced it.

The products formed by fermenting the mannitol have also been determined. The origin of this mannitol must be due to a reduction of fructose as a result of conditions incident to the fermentation process. On the basis of the experimental facts developed in this paper and the very complete data of Gayon and Dubourg and other investigators, it is suggested that the general line of reaction by which fructose is broken down and mannitol produced may be represented by the following equations.



If in the production of acetic and lactic acids from fructose nascent hydrogen is produced, this may then react with another fructose molecule and reduce it to mannitol. This theory does not regard mannitol as an intermediate but a coincident product in the formation of acetic and lactic acids. There is considerable evidence to support this theory in the papers of Gayon and Dubourg, Laborde, and Müller-Thurgau and Osterwalder. Carbon dioxide was always formed by their bacteria and malic acid was fermented to lactic acid and carbon dioxide by some of these organisms. Mannitol was found in quantities approximating what is required by this theory. Their organisms did not ferment mannitol and hence the theoretical amount of mannitol might accumulate. The data of Gayon and Dubourg, and Laborde are sufficiently complete to balance against that required by the above theory.

	Found.		Required.
	Gayon and Dubourg.	Laborde.	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Acetic acid.....	14.0	10.0	11.1
Lactic ".....	12.5	14.3	16.7
Mannitol.....	66.0	63.0	67.4
Carbon dioxide.....	9.0		8.2
Succinic acid.....	0.25		
Glycerol.....	0.60		

When we consider the difficulties and errors involved in the determination of some of these compounds, especially lactic acid, for which all methods probably give too low results, it is seen that fair agreement exists between the found and calculated values. The small amounts of glycerol and succinic acid found could also be accounted for as originating from the malic acid.

That the above theory does not seem to fit all the facts in respect to each mannitol-producing organism that has been isolated will be observed by a careful examination of the published data; but as a working hypothesis it may well serve for a time. The correlation of the above theory with the data obtained in the fermentation of fructose by the pentose fermenters will be considered in the experimental part of this paper.

EXPERIMENTAL.

In the fermentation of fructose, approximately 2 per cent solutions were used. The sugar was dissolved in a water extract of compressed yeast sterilized for 30 minutes and sterilized bromocresol purple added at the time of inoculation. The acids formed during the fermentation were neutralized with sterilized normal alkali whenever a decided acid reaction was indicated by the bromocresol purple. Controls, treated as above but not inoculated, were always run parallel with the fermenting culture solutions. The values obtained from the controls have been deducted in calculating the data given in the tables. At the end of the fermentation period the cultures were analyzed for acetic and lactic acids by the steam distillation and extraction methods employed in our previous work (1919). At the same time the

mannitol was determined either on an aliquot of the culture or, in a few cases, upon the ether-extracted residue. The procedure in general was that previously used by Gayon and Dubourg and others. The sample was first concentrated on the steam bath, filtered from any precipitate, and the filtrate evaporated to dryness on clean sand. This was then successively extracted with small portions of boiling 80 per cent alcohol until completely exhausted of mannitol. About ten extractions were usually made. In some of the later determinations an extraction apparatus similar in design to the Kutscher-Steudel extraction apparatus for non-volatile acids was used. The lower end of the tube was immersed in an oil bath kept at a temperature just below the boiling point of the alcohol. The extraction could thus be carried on continuously for any desired length of time. Usually the dried residue was extracted for about 10 hours. After the alcohol extract was filtered, it was concentrated to a thick syrup and set aside in the refrigerator for 1 or 2 days. Crystals usually formed over night, but in some cases crystallization was not complete until after 2 or 3 days. The crop of crystals was freed from the mother liquor by filtering through a hardened filter with the aid of suction, was washed with alcohol and finally with ether, then dried, and weighed. As thus obtained the mannitol crystals were usually snow-white. The mother liquor and washings were again concentrated to a thick syrup, placed in the refrigerator, and kept there until a second crop of crystals had separated out. Usually 2 or 3 days were required. Additional crystallizations were made until no more crystals formed even after standing in the ice box for 6 or 8 days. With the improved method of extraction, the recovery of a known quantity of mannitol from yeast water and the control solution was from 92 to 95 per cent.

The unfermented fructose in the culture was also determined but in no case did this exceed 0.1 gm. and in most of the cultures merely a trace of reducing sugar was found. As the cultures contained about 2 gm. of fructose when inoculated, it is clear that practically all the fructose had disappeared at this time.

The results of the analyses are given in Table I and disclose several interesting points:

The quantities of acetic acid and lactic acid are nearly equal, but in general with lactic slightly in excess of acetic.

The quantity of mannitol is greater in the young cultures than in the older ones. This is very clearly shown in the 22 and 51 day cultures of Nos. 41-11 and 118-8. These four cultures were made up from the same batch of medium and were grown under the same conditions so that the results obtained furnish a good basis for comparison. The mannitol has decreased

TABLE I.

Products Formed from the Fermentation of Fructose in 100 Cc. of Culture.

Time.	Culture No.	Weight of fructose.	Acetic acid.	Lactic acid.	Mannitol.	Ratio acetic: lactic.
<i>days</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
16	41-11	1.70	0.312	0.329	0.573	100 : 105
22	41-11	1.70	0.320	0.340	0.441	100 : 107
51	41-11	1.70	0.436	0.549	0.323	100 : 126
16	118-8	1.70	0.311	0.338	0.533	100 : 108
22	118-8	1.70	0.293	0.151	0.350*	
51	118-8	1.70	0.444	0.539	Trace.	100 : 125
31	41-11	1.70	0.360	0.333	0.443†	110 : 92
31	55-9	1.70	0.353	0.328	0.443†	100 : 93
31	69-19	1.70	0.288	0.347	0.443†	100 : 120
31	69-30	1.70	0.331	0.290	0.443†	100 : 90
31	118-5	1.70	0.312	0.347	0.443†	100 : 111
31	118-8	1.70	0.326	0.335	0.443†	100 : 102
20	55-9	1.70			0.629†	
20	69-19	1.70			0.719†	

* Low result is probably due to bone-blackening the alcohol solution before crystallizing.

† This figure was obtained by the analysis of the combined Cultures 41-11 to 118-8 inclusive.

markedly in both cultures, while the acetic and lactic acids have just as markedly increased. The fructose present in parallel cultures analyzed at the end of 22 days was less than 0.1 gm., so that the increase in the acids could not have come from unfermented fructose. It must therefore have originated from the fermentation of the mannitol. The mannitol is formed very early in the fermentation of the fructose and then is itself slowly attacked by the organisms. Further evidence on this point will be presented later.

The identification of mannitol is based principally upon its characteristic crystalline form and upon its melting point. Many determinations, made on the crystals isolated, gave figures ranging from 163–164°C. (uncorrected). A sample of commercial mannitol gave a value of 164°C. (uncorrected). The melting point of pure mannitol is given by various authorities as 166.05°C. (Braham). The crystalline appearance, melting point, and fermentability clearly establish the identity of the compound.

In the preceding experiments no attempt was made to determine the gaseous products involved. After some preliminary tests it was found that carbon dioxide was produced in considerable quantities. A number of experiments were therefore carried out in which the carbon dioxide was determined and a more complete accounting for the fructose fermented was obtained.

In these experiments the fermentation flask A (Fig. 1) was closed with a two-hole rubber stopper. Through one hole was passed a short glass tube B. The upper end was closed by means of a short piece of rubber tubing and a screw pinch-cock. The rubber tube was plugged with cotton and wrapped with tin-foil. When it became necessary to add alkali to the fermenting solution the tin-foil cap and cotton were removed, the pinch-cock was opened, and sterilized alkali run in from a sterilized, finely calibrated pipette.

Through the second hole in the rubber stopper a bent tube C made from a 25 cc. pipette was passed. The delivery end of this tube passed through a two-hole stopper into a small bottle D containing strong potassium hydroxide. A guard tube E containing soda lime was joined to the potassium hydroxide bottle to prevent absorption of carbon dioxide from the air. As a further precaution against leaks the rubber stoppers were coated with a mixture of beeswax and paraffin. This apparatus permitted the gas evolved in the fermentation flask to pass over into the bottle containing the potassium hydroxide where the carbon dioxide was absorbed and at the same time permitted the escape of the air contained in the bottle at the beginning of the fermentation. The bulb of the connecting tube prevented the potassium hydroxide from being sucked back into the fermentation flask, when the temperature changed or when fermentation ceased. At the end of the fermentation period, a small glass tube was passed through

B to the bottom of flask A and the greater part of the CO_2 remaining in flask A was aspirated over into D by means of a current of CO_2 -free air. The carbon dioxide in the culture solution and in the potassium hydroxide was determined by means of Van

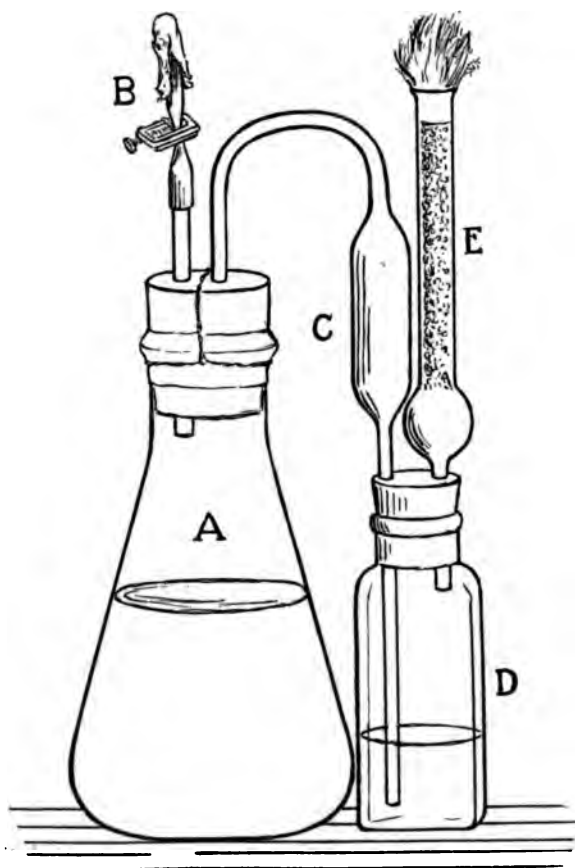


FIG. 1. Fermentation flask with CO_2 absorption bottle.

Slyke's apparatus for determining carbon dioxide in blood and carbonate solutions. The data obtained are given in Table II.

From the results of Table II it is seen that about 85 per cent of the fermented fructose is accounted for by the products acetic acid, lactic acid, mannitol, and carbon dioxide. The iodoform

test for alcohol was negative and the result by the potassium dichromate method for the quantitative determination of alcohol showed little more volatile acid than the reagents alone. The data obtained from the analyses of the 4 day cultures of No. 118-8 bring out very clearly the rapidity with which fructose disappears from the culture solution; less than 0.1 gm. of fructose remained unfermented. About one-half the sugar consumed had been broken down into acetic and lactic acids and carbon dioxide. A somewhat smaller proportion of the sugar had been converted into the mannitol. The evidence indicates that the mannitol is not attacked until the more easily fermented fructose has been consumed. As will be shown later the slow rate of fermentation of mannitol lends support to this view.

TABLE II.

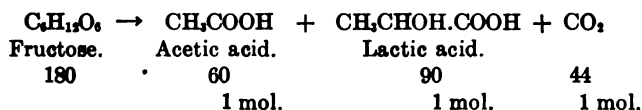
Total Fermentation Products Formed and Per Cent of Fructose Accounted for by the Products.

Time.	Culture No.	Weight of fructose fermented.	Acetic acid.	Lactic acid.	Mannitol.	Carbon dioxide.	Fructose accounted for by products.
days		gm.	gm.	gm.	gm.	gm.	per cent
17	41-11	1.70	0.326	0.365	0.570	0.215	87
17	118-8	1.60	0.266	0.284	0.452	0.220	77
4	118-8	1.60	0.239	0.260	0.764	0.176	90
58	118-8	1.60	0.298	0.180	0.450	0.273	75

Due to the improved means of extraction already described, the values for mannitol obtained here are higher than in the earlier experiments.

From the data in Table II the molecular proportions of the several products have been calculated and appear in Table III.

The molecular ratios are in fair harmony with the reaction previously proposed.



The only serious departure is in the case of lactic acid. This is not unexpected since all the experimental errors tend to give a low value for lactic acid.

The possibility is always present that the lactic acid formed from the fructose may in turn be fermented subsequent to the fermentation of the fructose itself. This possibility is strongly indicated by the data obtained from the analysis of the 58 day culture. The data obtained from the fermentation of malic acid and old cultures of mannitol lend considerable support to this possibility. Kayser described a type of fermentation in which acetic acid and lactic acid were produced; but he believed that when fermentation was continued for some time the lactic acid was almost completely converted into acetic acid.

TABLE III.
Molecular Relations of the Fermentation Products of Fructose.

	Culture 41-11.			Culture 118-8.		
	Acetic acid.	Lactic acid.	CO ₂	Acetic acid.	Lactic acid.	CO ₂
Weights divided by molecular weights.....	0.051*	0.041	0.050	0.044*	0.031	0.050
Molecules.....	1.0	0.8	1.0	1.0	0.7	1.1
Weights divided by molecular weights.....				0.040†	0.029	0.040
Molecules.....				1.0	0.7	1.0
Weights divided by molecular weights.....				0.050‡	0.020	0.062
Molecules.....				1.0	0.4	1.5

* After 17 days fermentation.

† " 4 "

‡ Same culture as (†) analyzed 54 days later.

Fermentation of Malic Acid.

In order to test the hypothesis that malic acid may be an intermediate product in the fermentation of fructose by the pentose fermenters the fermentability of malic acid was demonstrated experimentally and the products formed were determined. In Series I a solution of malic acid was neutralized with sodium hydroxide and then made up with yeast water to a volume equivalent to 2 per cent calculated as malic acid. These solutions were inoculated with Cultures 41-11 and 118-8, and sterilized bromocresol purple was added. Fermentation of the sodium malate

was shown by the solutions becoming alkaline to the bromocresol purple. This change indicates the decarboxylation of the salt, the formation of sodium bicarbonate, and the production of a mono-basic acid instead of a di-basic acid. The alkalinity thus developed soon checks the activity of the organisms. The cultures were therefore neutralized with 0.1 N malic acid at intervals of about 2 days each. That malic acid ferments very rapidly may be seen from the curve of acid production given in Fig. 2 at the end of this paper. The rate of acid production was somewhat slower at first than was the case with fructose, but soon became faster and continued at this very rapid rate to the end of the

TABLE IV.
Fermentation of Malic Acid Salts Calculated for 100 Cc. of Culture.

Culture No.	Length of fermentation.	Weight of malic acid.	Volatile acid as acetic.	Non-volatile acid as lactic.	Carbon dioxide.
	days	gm.	gm.	gm.	gm.
Series I.					
41-11	30	3.140*	0.146	0.804	Undetermined.
118-8	30	3.140*	0.176	0.734	"
Series II.					
41-11	16	0.684*	0.078	0.342	0.260
118-8	16	0.684*	0.070	0.268	0.216
Series III.					
41-11	23	2.000†	0.043	0.113	0.084
118-8	23	2.000†	0.059	0.111	0.102

* As the neutral sodium salt.

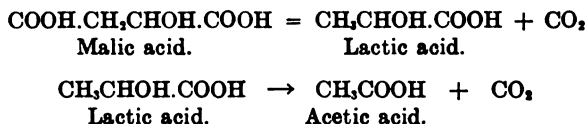
† " " " calcium "

fermentation period. The malic acid thus fulfills one of the requirements for an intermediate fermentation product; viz., that the intermediate product must ferment as fast as or faster than the compound from which it is formed. The results of the malic fermentation are given in Table IV.

In Series II and III the carbon dioxide evolved was determined. Except in Series II, where only small amounts of malic acid were used, large amounts of malate remained unfermented. In Series I fermentation ceased because of the high alkalinity developed, caused by the inevitable accumulation of sodium bicarbonate. In Series III the alkalinity must be due to calcium

carbonate which is not injurious to the growth of the organisms. Some other factor must therefore have operated to check the fermentation in this case.

When the cultures were analyzed it was found that an appreciable amount of volatile acid had been developed. This acid was later identified by its Duclaux distilling constant as almost entirely acetic. The quantity of carbon dioxide found is greater than that required on the assumption that one molecule of carbon dioxide is formed for one molecule of lactic acid. The production of acetic acid and an excess of carbon dioxide, therefore, indicates that some change other than the simple malic to lactic takes place. The origin of the acetic acid and the excess of carbon dioxide might be explained on the assumption that a secondary fermentation of the lactic acid follows the primary malic to lactic fermentation. On this hypothesis the breaking down of the malic acid may be represented schematically.



This is only a working hypothesis and is subject to more complete experimental data.

The possibility of organic acids other than malic being intermediate products in the fermentation of fructose was tested, but none was found that could be fermented. The malic acid cultures were tested for alcohol, but none was found.

Fermentation of Mannitol.

In the course of a study on the pentose fermenters it was found that mannitol was broken down with the formation of acetic and lactic acids. An experiment was therefore set up to test the fermentability of the mannitol, formed from fructose, by the same bacteria that produced it. For this purpose 40 gm. of fructose were dissolved in 2 liters of water and 100 cc. portions placed in twenty flasks. Ten flasks were inoculated with Culture 41-11 and ten with Culture 118-8. The mannitol produced from 30 of the 40 gm. of fructose was isolated as

previously described and amounted to 5.97 gm. Culture solutions containing 2 per cent of this mannitol were then inoculated with the six pentose fermenters previously studied and the acid production was roughly measured by the addition of normal alkali from time to time. After varying lengths of time the cultures were analyzed for acetic and lactic acids. The data obtained together with that from the fermentation of commercial mannitol are given in Table V.

TABLE V.

Acid Production from Fermentation of Cultures Containing 2 Per Cent Mannitol. Calculated for 100 Cc. of Culture.

Time.	Culture No.	Mannitol used.	Volatile acid as acetic.	Non-volatile acid as lactic.	Ratio acetic: lactic.
<i>days</i>			<i>gm.</i>	<i>gm.</i>	
18	41-11	Merck's C.P.	0.185	0.221	100:120
18	55-9	"	0.190	0.232	100:123
18	69-19	"	0.146	0.230	100:158
18	69-30	"	0.151	0.184	100:122
18	118-5	"	0.155	0.175	100:113
18	118-8	"	0.137	0.194	100:142
66	41-11	"	0.560	0.788	100:141
37	55-9	"	0.325	0.522	100:160
66	69-19	"	0.317	0.497	100:156
60	118-8	"	0.266	0.360	100:135
42	118-8	From fructose.	0.415	0.499	100:120
87	41-11	" "	0.798	0.306	100: 38
87	55-9	" "	0.544	0.228	100: 42
38	69-19	" "	0.352	0.507	100:144
38	69-30	" "	0.239	0.305	100:128
38	118-5	" "	0.224	0.282	100:126

An examination of the data shows a slow but steady fermentation. After 18 days the acids formed represent about 20 per cent of the initial mannitol and at the end of 66 days this has risen to as high as 67 per cent in the case of Culture 41-11. Only slight differences in the vigor of the cultures are apparent during the early stages of the fermentation, but at the end more marked differences appear. Cultures 41-11, 55-9, and 69-19 show conspicuous activity in the fermentation of both mannitol and fructose. The ratio of acetic to lactic runs much the same throughout the period of fermentation except in the two 87 day

Cultures 41-11 and 55-9, where a very pronounced change in the ratio has taken place. Apparently in these two cases some of the lactic acid has been broken down into acetic acid. This possibility was also pointed out in connection with the fermentation of fructose, Tables II and III, malic acid, Table IV, and in the discussion of Kayser's work on the fermentation of glucose.

The commercial c. p. mannitol and the mannitol from fructose are fermented in exactly the same manner. The rate of fermentation, the products formed, and the ratio of the two acids are alike in both cases. Biologically, as well as chemically, the mannitol from fructose appears to be the same compound as the commercial product.

Identification of the Volatile Acids.

The volatile acids formed from the fermentation of fructose, mannitol produced from fructose, Merck's c. p. mannitol, and malic acid were subjected to a Duclaux distillation and their distilling constants determined. The data obtained in a few representative cases are given in Table VI.

It is evident from this table that the volatile acid formed by the fermentation of fructose mannitol, whether the commercial or biological product, and of malic acid is practically all acetic acid. The agreement between the distilling constants obtained and that required for acetic is very good. In a few representative cases the barium content of the acids used in the Duclaux distillation was determined subsequent to this distillation, and serves further to identify the volatile acid as nearly pure acetic. The data are given in Table VII.

Identification of the Non-Volatile Acid.

The non-volatile acid was identified as lactic acid by its barium salt and zinc salt. The free acid was extracted by the usual ether extraction method and titrated with barium hydroxide; from the barium salts the zinc salts were made. The zinc lactate crystals thus obtained had the crystalline appearance of the inactive or racemic type (Dox and Neidig). The determination of the water of crystallization showed that three molecules of water were present for each molecule of salt. The data obtained are given in Table VIII.

TABLE VI.

Distilling Constants of the Volatile Acids Obtained by Duclaux Method.

Source of acid.	Culture No.	Fractions.									
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Fructose.....	41-11	7.1	15.8	24.3	33.1	42.2	51.8	62.0	73.0	85.2	100
“.....	118-8	7.1	16.4	24.8	33.5	42.6	52.1	62.3	73.3	85.5	100
Mannitol from fructose.....	69-19	7.5	15.5	24.0	32.7	41.9	51.6	62.0	73.0	85.0	100
Mannitol (Merck's c.p.).....	41-11	7.4	15.3	24.0	32.2	41.6	51.3	61.5	72.5	85.2	100
Malic acid.....	41-11	7.8	15.9	24.2	32.8	41.8	51.2	61.4	72.5	84.9	100
Duclaux constant for acetic acid....	and 118-8.	7.8	15.9	24.2	32.8	41.8	51.2	61.4	72.5	84.9	100
		7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100

TABLE VII.

Composition of the Barium Salts of the Volatile Acid.

Compound fermented.	Culture No.	Barium salts of the volatile acid.	Barium sulfate equivalent.	
			Found.	Calculated for acetic acid.
		gm.	gm.	gm.
Fructose.....	118-8*	0.3854	0.3488	0.3521
“.....	118-8†	0.7566	0.6816	0.6913
Mannitol from fructose.....	41-11	0.3314	0.3030	0.3028
“ (Merck's c. p.).....	41-11	0.5748	0.5151	0.5234
Sodium malate.....	41-11 and 118-8.	0.3416	0.3048	0.3121

* Fermented 4 days.

† “ 58 “

TABLE VIII.

Water of Crystallization Contained in Zinc Lactate.

Source of salt.	Culture No.	Zinc lactate used.	Water lost.		Water in Zn (C ₃ H ₅ O ₃) ₂ + 3H ₂ O.
			gm.	per cent	
Fructose.....	118-8, 3 days old.	0.1472	0.0260	18.2	18.17
“.....	118-8, 12 days old.	0.1186	0.0208	17.6	18.17
Mannitol from fructose...	69-19	0.3022	0.0602	19.1	18.17
“ (Merck's c.p.)...	41-11	0.464	0.0824	17.8	18.17

In a few cases the non-volatile acid was examined for its barium content. After the ether extract was titrated with barium hydroxide, the solution of barium salts was evaporated to about 10 cc., made up to 100 cc. with 95 per cent alcohol, filtered from any precipitate, and an aliquot evaporated to dryness in a platinum dish. The salts were dehydrated by heating at 130°C. for several hours until constant weight was secured and then were converted into barium sulfate by burning off the salts in the presence of an excess of sulfuric acid.

From the data thus obtained it is apparent that the non-volatile acid is almost wholly lactic acid. No evidence of succinic or other acids was obtained. The slightly lower values found are

TABLE IX.
Lactic Acid Produced from Fructose and Its Related Compounds.

Compound fermented.	Culture No.	Weight of barium salts.	Weight of barium sulfate.	
			Found.	Calculated.
		gm.	gm.	gm.
Fructose.....	41-11	0.1630	0.1176	0.1206
"	118-8	0.1640	0.1206	0.1214
Mannitol from fructose.....	118-5	0.3128	0.2238	0.2315
" (Merck's C.P.).....	118-8	0.5466	0.3900	0.4044
Malic acid.....	41-11	0.5386	0.3912	0.3985
" "	118-8	0.4862	0.3564	0.3597

due to the presence of a small amount of soluble organic matter, which was produced in the sterilization of the sugar and was weighed together with the barium lactate. The data are given in Table IX.

Rate of Acid Production.

As measured by the production of acid, fructose is very readily fermented by the pentose fermenters. The rate of acid production is not so fast as in the case of xylose and arabinose, but much faster than from glucose or galactose. The acidity of the fermenting solution, as indicated by the bromocresol purple, was neutralized from time to time by the addition of normal alkali. In this way the rate of development of acidity was noted. In Table X

are given the cc. of 0.1 N alkali or acid added at different times to cultures of fructose, commercial mannitol, mannitol obtained from fructose, and malic acid salts. From fructose approximately 40 cc. of 0.1 N acid were produced within 3 days, while from mannitol less than one-half this amount was produced. The fermentation in the case of fructose starts off quickly and is conspicuously faster for 15 to 20 days than in the case of mannitol. At the end of 23 days the quantity of acid produced from the two materials is nearly equal.

TABLE X.

*Rate of Acid Production from Fructose, Mannitol, and Sodium Malate.
Total 0.1 N Acid in 100 Cc. of Culture.*

Time.	Fermentation of fructose.				Fermentation of Merck's mannitol.				Fermentation of mannitol from fructose.				Fermentation of sodium malate.	
	Culture No.				Culture No.				Culture No.				Culture No.	
	41-11	55-9	69-19	118-8	41-11	55-9	69-19	118-8	41-11	55-9	69-19	118-8	41-11	118-8
days	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
3	40	36	31	40	25	12	5	9	6	7	10	6	10	10
6	65	65	53	67	58	27	31	22	22	23	26	20	50	50
10	80	80	68	76	70	45	51	37	37	40	42	30	90	90
18	90	88	80	85	85	69	64	53	61	73	63	53	145	145
27	103	101	92	96	106	91	75	66	82	102	84	75	180	180
38									100	128	106	100		
51									124	136				
63									147	142				

The conversion of fructose into mannitol is much more rapid than the breaking up of the latter into acetic and lactic acids and hence results in the accumulation of the mannitol. It is possible that no mannitol is attacked until most of the fructose has been used, because the latter compound is more readily utilized than the former. If the formation of mannitol is merely incidental to the strong reducing conditions set up in the fermentation of the fructose, then the mannitol would probably not be attacked until all the fructose had been consumed.

The rate of fermentation of the sodium malate, as judged from the production of acid, is equal to that of the fructose itself, and continues at this high rate throughout the fermentation period.

From the data in Table X four composite curves have been constructed to represent the average rate of acid production from the compounds fermented. The average of the figures for all the cultures at any given time has been taken to represent the acid produced at that particular time from each of the above four types of fermentation. Examination of Fig. 2 shows the general progress of the fermentation.

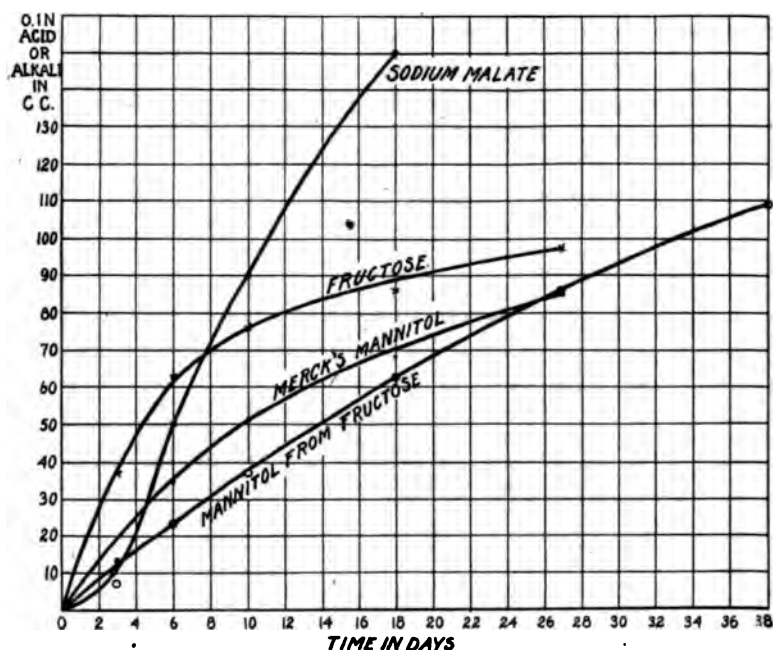


FIG. 2. Curves show average rate of acid production from different substances.

SUMMARY.

1. Fructose is readily fermented by *Lactobacillus pentoceticus*, *n. sp.*, with the production of acetic acid, lactic acid, mannitol, and carbon dioxide.
2. The mannitol accumulates in considerable quantities, representing 30 to 40 per cent of the fructose in the early stages of the fermentation. It has the crystalline appearance and the same

melting point, 164°C. (uncorrected), as the commercial c.p. product.

3. With the mannitol thus obtained a culture medium can be made and fermented by the same bacteria that produced the mannitol. The products formed are acetic acid and lactic acid. The fermentation of this mannitol is identical with that of commercial mannitol both with respect to the rate of fermentation and the end-products formed.

4. If the fructose solution is allowed to ferment for a long time the mannitol, which accumulates at first, undergoes slow fermentation; acetic and lactic acids are produced and eventually all the mannitol is destroyed.

5. The production of acetic and lactic acids from fructose is very rapid during the first days of the fermentation but slows up after 10 days. All the fructose disappears within 5 days. The fermentation of mannitol, on the other hand, is slow in starting but continues at about the same rate throughout the entire period. This difference in the fermentation of the two compounds is explained by assuming that the strong reducing conditions set up by the rapid production of acetic and lactic acids quickly convert the unfermented fructose into mannitol. Within a few days the fructose has thus been used up in the production of acetic acid, lactic acid, carbon dioxide, and mannitol. The slower fermentation which then ensues is really the decomposition of mannitol.

6. Sodium and calcium malate are fermented by the same organisms and yield chiefly lactic acid and carbon dioxide and a small quantity of acetic acid as the end-products.

7. On the basis of the above facts it is suggested that the main line of the fermentation process is the production of acetic acid, lactic acid, and carbon dioxide. It is assumed that malic acid is an intermediate compound in this process. The strong reducing conditions incident to this fermentation result in the reduction of a large part of the unfermented fructose to mannitol. This mannitol is subsequently attacked; and thus a second fermentation entirely different from that of fructose is set up.

8. Some evidence is presented to indicate that in a fermentation extending over a long period lactic acid itself is broken down to acetic acid by the pentose-fermenting bacteria.

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NUTRITIVE FACTORS IN PLANT TISSUES.*

III. FURTHER OBSERVATION ON THE DISTRIBUTION OF WATER-SOLUBLE VITAMINE.

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(Received for publication, January 17, 1920.)

In continuation of our earlier efforts to secure a more accurate comparison of the content of water-soluble vitamine of natural foods¹ the following method was employed with a series of plant products. Alfalfa, cabbage, clover, spinach, and timothy were dried in the manner already described.¹ Beet, carrot, and turnip roots were washed, cut into thin slices, desiccated in an air drier at 50–60°, and ground finely. Commercially canned tomatoes were dried at 60–70° and ground. Whole potato consisted of unpeeled potatoes boiled until tender, drained, mashed or sliced, dried at 60–70°, and ground. Peeled potato was prepared by paring raw potatoes as in domestic practise, boiling them until tender, mashing without draining, drying at 60–70°, and grinding. Furthermore, to compare the parts of the entire potato tuber with respect to the distribution of the water-soluble vitamine the potato peel was boiled in distilled water until tender, dried without draining, and ground.

These dried products were fed, apart from the rest of the food, to growing white rats receiving a diet adequate for them except

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington.

¹ Osborne, T. B.,[†] and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187; xxxix, 29.

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in respect to the lack of water-soluble vitamine. The basal food mixture consisted of:

	<i>per cent</i>
Meat residue.....	19.6
Salt mixture*.....	4.0
Starch.....	52.4
Butter fat.....	9.0
Lard.....	15.0

* For composition of salt mixture see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

The weekly food intake was determined except in a few instances where the animals spilled their ration and made the estimation inaccurate.

There are two ready methods of testing products for the presence of water-soluble vitamine. One consists in feeding the material to be investigated to animals that have declined on a diet deficient in this food factor. Such tests, however, will not demonstrate the *comparative* vitamine content of different foods. They are essentially restorative in character. If the outcome is positive in the sense of a renewal of nutritive well being, the test for the vitamine may be qualitatively successful. However, animals which have suffered a vitamine deficiency may be so badly malnourished or underfed that relatively excessive amounts of the vitamine-bearing food may be required to restore them to normal condition. In that event a failure to promote nutrition by the extract to be tested may be ascribed to the condition of the animal rather than the product. Comparisons of vitamine-bearing foods may not be reliable when they involve attempts to restore nutrition in animals that have suffered to a variable degree from unlike deficiencies.

The other method consists in feeding the supposed source of vitamine to animals which are normal in health and development, and observing whether the product offered supplies what is needed to promote normal growth when the diet affords an adequate supply of all essentials except that to be investigated. This procedure has been adopted in the present experiments. The basal ration included fat-soluble vitamine in the form of butter fat (9 per cent), but was devoid of water-soluble vitamine. Meat resi-

due, prepared in the manner already described by us² was selected as the source of protein because our earlier experiments showed this product to be practically free from the vitamine which it was here proposed to investigate. On the basal ration selected, growing rats of 60 to 120 gm. in body weight begin to decline in weight within a few days. Their food intake decreases and they invariably die within 80 days unless a source of water-soluble vitamine is furnished.³ We fed the dried vegetable to be tested apart from the basal ration, so that the dosage might be accurately ascertained. With few exceptions the rats ate all the dried products each day; the data for this factor consequently have a strictly quantitative value in the comparisons between different tests.

Starting with healthy growing rats approximating 90 gm. in body weight the experiments were extended over 8 weeks with each dried food. The daily doses of the latter were varied, 1 and 0.5 gm. portions—in some cases 0.2 and 2 gm. doses—being tested.⁴ At the end of this period, especially when growth had not proceeded at the normal rate, many of the animals were tested for a further period of 6 weeks with our standard mixed food or with daily additions of 0.2 gm. of dried brewery yeast⁵ to determine whether the failures to grow satisfactorily had actually been due to lack of water-soluble vitamine rather than some other incidental factor.

The outcome of these comparative tests is shown in Charts I to X. In these experiments *alfalfa* and *clover* surpassed all the other nine products tested in equal doses as sole sources of water-soluble vitamine. Several of the animals (Rats 5769, 5768, 5775, 5790, Charts I and II) gained 175 gm. or more in weight during the 8 weeks period—a growth equal to the best which we have observed in our stock colony. This is a result far better than we

² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309.

³ Osborne, T. B., Wakeman A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

⁴ The portions used were measured by means of small, calibrated scoops which were designed to deliver them in fairly accurate amounts. However, in the course of the experiments it was discovered that when the atmospheric humidity was unusually high the dosage of the dry powders furnished in this way was sometimes only approximately correct.

⁵ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.

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have ever secured with even 16 cc. of milk per day fed as a source of water-soluble vitamine in addition to the same basal ration. Half of this allowance, *i.e.* 0.5 gm. per day, of dry alfalfa or clover is not equally efficient (Rats 5744, 5750, 5723, 5772, 5770, 5774, Charts I and II).

Tomato is rich in water-soluble vitamine. 1 gm. daily doses promoted good growth when the animals would eat this quantity (Rats 5928, 6008, Chart III). At first it was frequently refused, perhaps because of the acrid flavor of the thus concentrated, highly acid product. 0.5 gm. doses of dried tomato were by no means so effective (Rats 5878, 5892, 5871), yet even 0.2 gm. quantities occasionally promoted limited growth or at least maintenance.

Spinach, cabbage, turnip, and carrot were not widely unlike in their comparative potency as sources of water-soluble vitamine. 1 gm. doses did not surpass 0.5 gm. quantities of alfalfa and clover (and perhaps tomato) in the results attained; whereas with the smaller amounts growth was not maintained (Charts IV to VII). The *beet root* (Rats 5972, 5980, Chart VI) did not equal the other roots tested. This is in harmony with the outcome of the observations of Steenbock and Gross⁶ on the sugar beet and mangels, in contrast with the carrot, rutabaga, and dasheen. The *timothy* hay though potent in the earlier periods of the experiment (Rats 5931, 5867, 5865, Chart VIII) proved disappointing in the long run, in view of the expectations raised by the brief trials already reported.⁷

The *potato* is evidently as rich in water-soluble vitamine as some of the roots tested, although similar quantities of the leguminous hays, alfalfa and clover, or the tomato promoted more rapid growth. Compared in 1 gm. daily doses there was little if any advantage in potato retaining the outer layers (Rats 5954, 6021, 6017, Chart IX), over peeled potato of the same age (Rats 5961, 5955, 5978, Chart X). This corresponds with the observation that the dried *potato peel* (Rats 5963, 6037) is no richer in the vitamine than are corresponding quantities of whole potato (Rats 5926, 5975, 5977, 6042). Even 2 gm. daily doses of *peeled potato*

⁶ Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.

⁷ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxix, 29, Chart II.

(Rats 5981, 5984, 5985) did not promote growth at the normal rate during the entire 8 weeks period.

Hess and Unger⁸ reported a slight difference in the antiscorbutic properties of old and fresh carrots. The comparative tests which we have made with *old* and *new* potatoes respectively in both 1 gm. and 0.5 gm. daily additions have not given indications of any noteworthy differences in content of the water-soluble vitamine here under investigation (Rats 5954, 6021, 6017, 5926, old potato; Rats 6082, 6044, 6086, 5975, 5977, 6042, new potato).

Bachmann⁹ has reported that certain yeasts will not grow on synthetic media unless organic material is added in small amounts. The substances which when added to Nägeli's solution made it possible for the yeast to cause fermentation were said to be such as "have been found to be rich in vitamines, especially water-soluble B." Extracts were prepared from potato, carrot, celery, beet, onion, rutabaga, cabbage, cauliflower, parsnip, and lettuce. "All the extracts furnished the necessary substance for the yeast to grow and ferment the medium even though the amount added was very little . . . the extracts were not equally potent." It is suggested by Bachmann that the method might be used to determine the presence of certain vitamines and even to determine quantitatively the amount present in various substances.

The statistical facts—total gains in weight and total food intakes in the 8 weeks period during which the dried plant products were fed by us in comparable doses each day to rats—are summarized in Table I.

As might be expected the greatest gains were in general the outcome of food intakes large in comparison with those of the rats which made smaller increments of weight. This is conspicuous in comparing the food intake and gains after feeding different amounts of the sources of vitamine. It should be noted that the figures recorded in the table as "gain" represent the end result of 8 weeks tests in the course of which a number of the rats, as shown in the charts of growth, reached larger size and subsequently declined somewhat owing, presumably, to the inadequate supply of vitamine. If the food intakes in cases of the smaller gains seem

⁸ Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1919, xvi, 52.

⁹ Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

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TABLE I.
*Total Gains in Body Weight and Food Intake.**
8 Weeks Period.

Food.	1 gm. daily.			0.5 gm. daily.		
	Rat No.	Gain in weight.	Total food intake.	Rat No.	Gain in weight.	Total food intake.
		gm.	gm.		gm.	gm.
Alfalfa.	5769	200	655	5744	144	536
	5768	179	594	5750	121	499
	5868	146	562	5723	106	516
Clover.	5775	197	663	5774	112	489
	5790	177	565	5770	102	441
	5791	150	497	5772	99	448†
Tomato.	6008	122	543	5878	88	453
	5936	120	516	5892	70	484
	5932	118	468			
	5928	115	544			
Spinach.	5870	119	476†	5808	87	439
	6014	101	445	5817	61	364
	5767	81	395	5857	32	326
	5914	75	456			
Cabbage.	5907	99	472	5922	52	314
	5921	91	364	5918	49	314
	6009	58	472	5919	30	271
Turnip.	5920	96	406	5842	46	306
	5869	68	268	5895	30	312
	5820	66	347†	5839	20	258
Carrot.	5824	78	398	5819	49	334
	5816	78	439†	5805	38	306
	6016	66	393	5802	36	287
	5758	46	398			
Timothy.	5867	42	367	5912	22	261
	5931	40	342	5970	5	317†
	5865	10	314	5909	-2	214

TABLE I—*Concluded.*

Food.	1 gm. daily.			0.5 gm. daily.		
	Rat No.	Gain in weight.	Total food intake.	Rat No.	Gain in weight.	Total food intake.
		gm.	gm.		gm.	gm.
Peeled potato.	5961	63	355			
	5978	52	367			
	5955	45	358			
Whole " old.	6021	60	439	5926	11	269
	5954	57	351			
	6017	40	368			
" " new.	6086	83	366	5977	25	296
	6082	54	361	5975	5	306
	6044	43	362	6042	4	238
Potato peel.				6037	4	225
				5963	4	267
Beet root.	5980	6	240			
	5972	-9	246			
0.2 gm. daily.						
Tomato.	5862	51	321			
	6018	32	347			
	6023	30	305			
	5866	2	255			

* The food intake is exclusive of the dried vegetable supplied.

† Some of the food was spilled by the rat so that the data are not entirely accurate.

disproportionately large it must be recalled that even when no growth whatever occurs a maintenance requirement must be met. In comparing the quota of the food devoted to growth, the maintenance requirement ought strictly to be subtracted from the total intake, and comparisons made on the basis of the difference. Inasmuch as the maintenance needs vary at different ages this calculation cannot be satisfactorily carried out with the data available. In earlier experiments with foods of similar make-up

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we¹⁰ found male rats, beginning at 90 gm. in weight, to make average gains of approximately 85 gm. in 8 weeks on a food intake of about 450 gm. Many of the animals in the present series have given even better results.

Inasmuch as the daily allowance of water-soluble vitamine was fixed, whereas the food intake varied with the individual and still more with the character of the plant product fed, the ratio of the latter to the total food eaten varied. In the case of rats receiving 1 gm. portions the vitamine-bearing vegetable product ranged from 10 per cent of the food intake or less in those which grew best, to 15 per cent or more at the other extreme of the series. In the 0.5 gm. experiments the content of plant food in relation to the total food ranged from about 5 to 9 per cent. Thus, the growth during the period when the dried tomato fed was equivalent to about 5 per cent of the food was greater than that in animals for which carrots supplied nearly three times this proportion. Dried brewery yeast is effective in far smaller proportions.⁵ The method is not accurate enough and the statistics not sufficiently abundant to permit more than the approximate comparisons already referred to.

EXPLANATION OF CHARTS.

The following charts represent the growth of rats beginning with a body weight of approximately 90 gm. during a period of 8 weeks in which the sole source of water-soluble vitamine was supplied by the indicated quantities of various dried vegetable tissues, fed daily apart from an otherwise adequate standard food mixture. At the end of the 8 weeks feeding trial the growth capacity of the animals was further tested by feeding 0.2 gm. of dried brewery yeast in place of the dried vegetable, or, in a few cases, substituting the ordinary mixed food of our stock colony for the previous diet. The duration of the yeast feeding is indicated by the interrupted line (— — — —) and the mixed food by the designation (—:—:—:—). Days on which animals failed to eat all the dried vegetables offered are indicated by asterisks on the curve. The statistics for food intake, etc. are summarized in Table I.

¹⁰ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

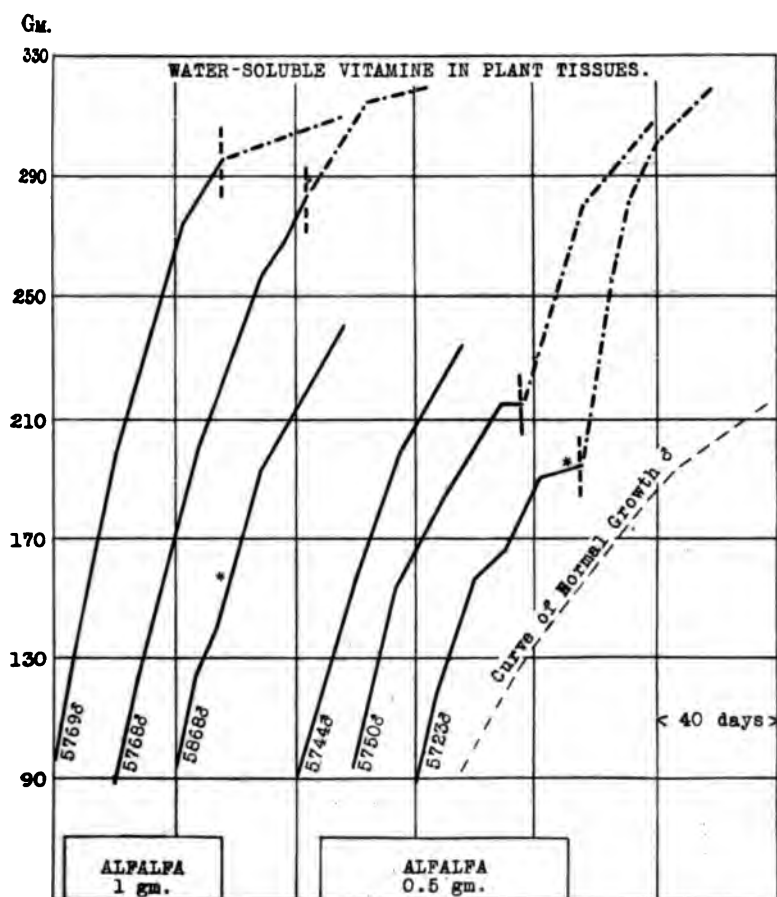


CHART I.

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Gm.

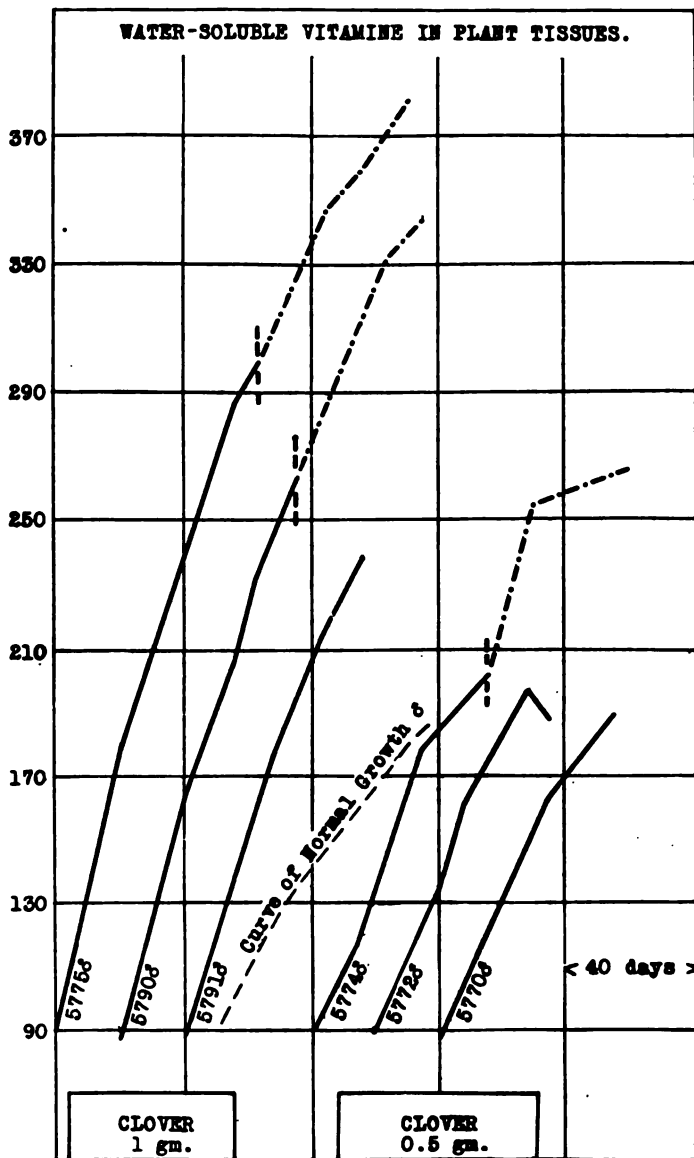
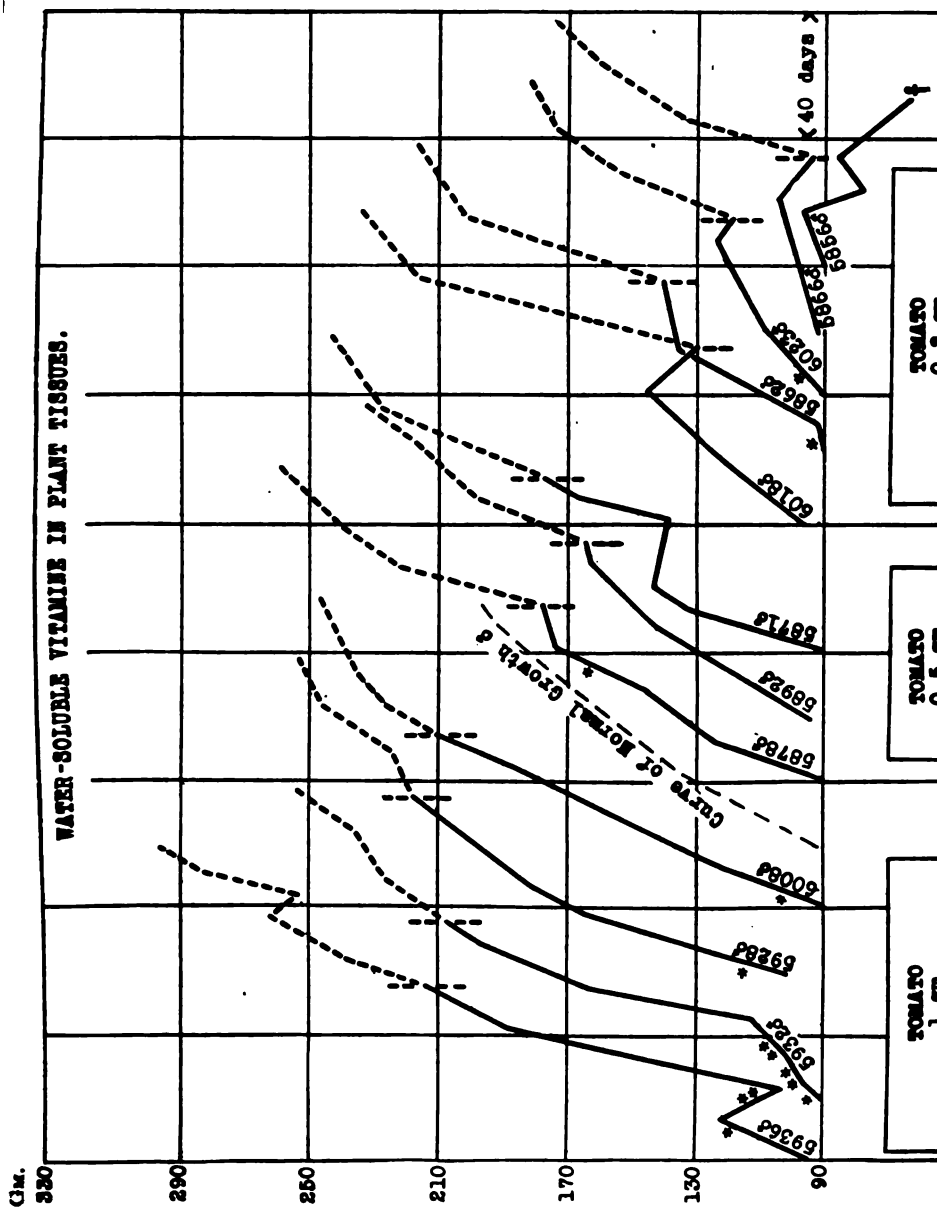


CHART II.



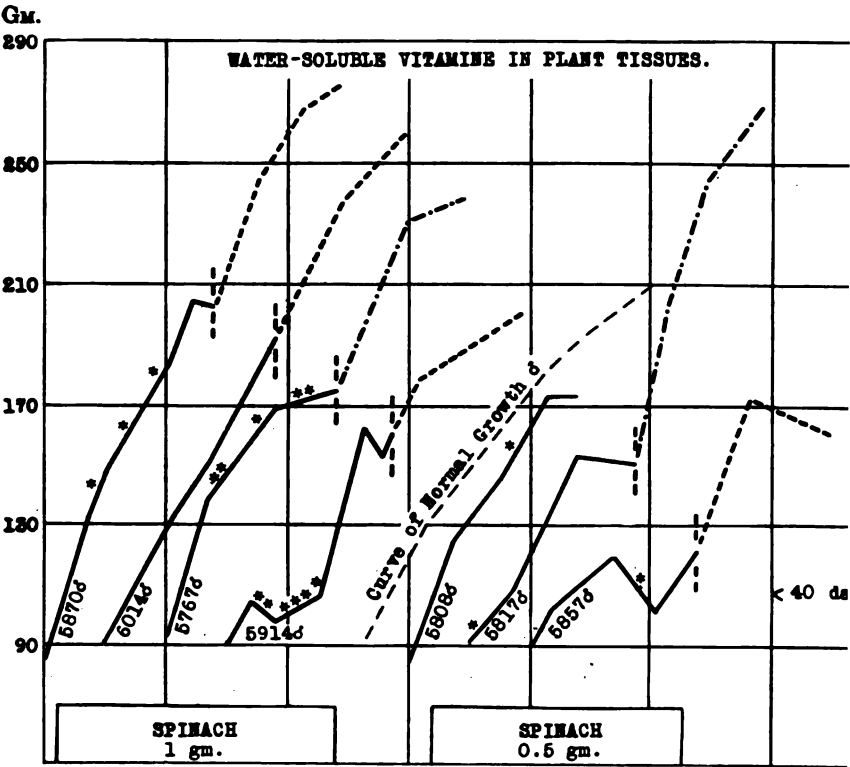


CHART IV.

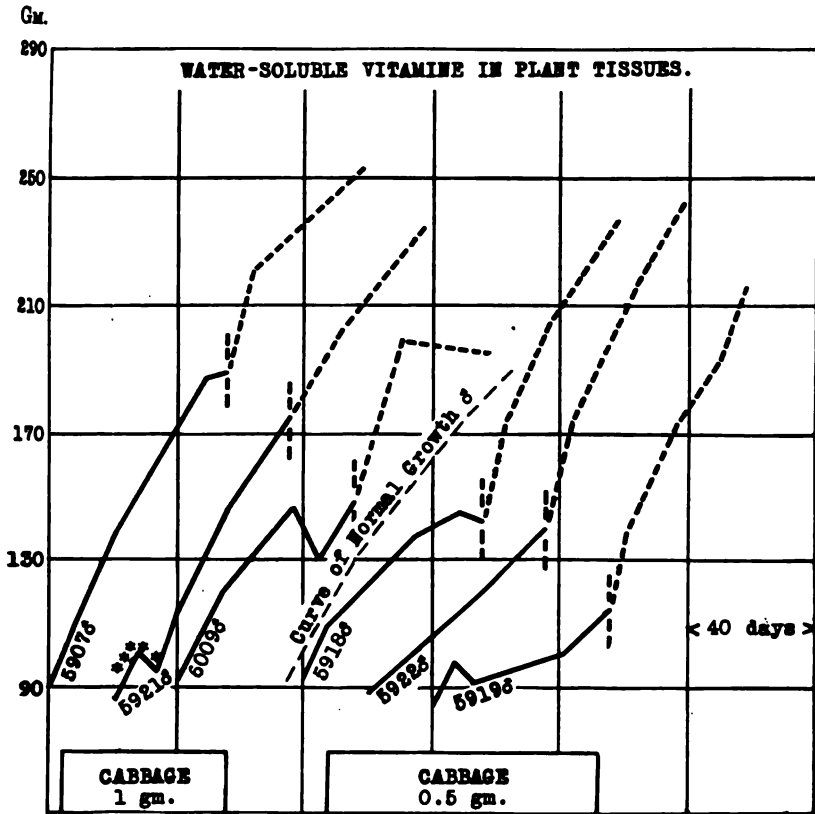


CHART V.

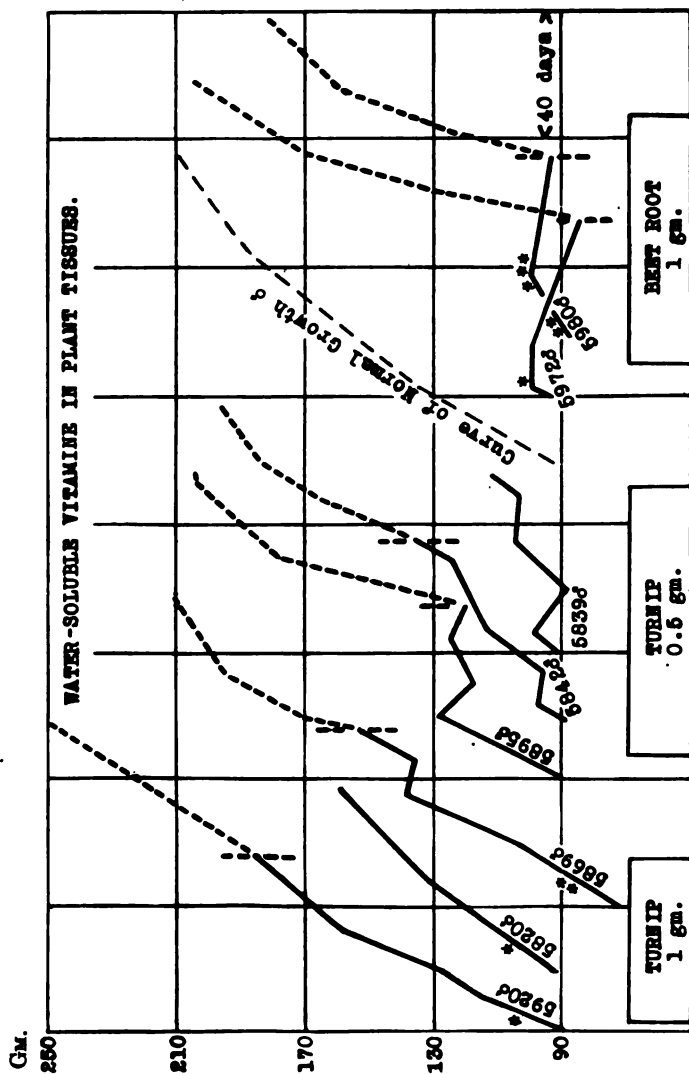


CHART VI.

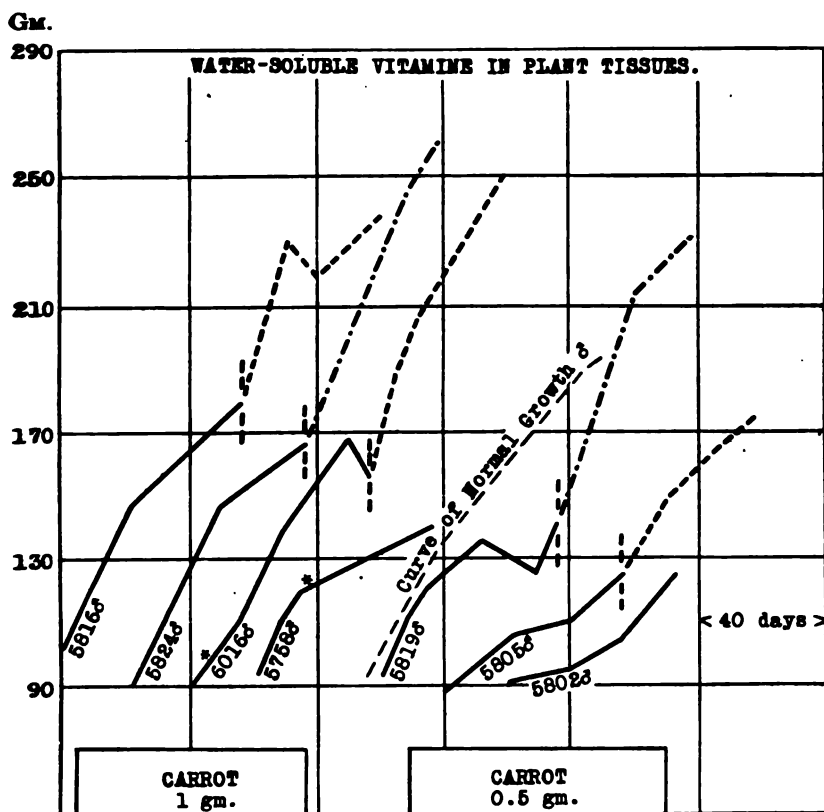


CHART VII.

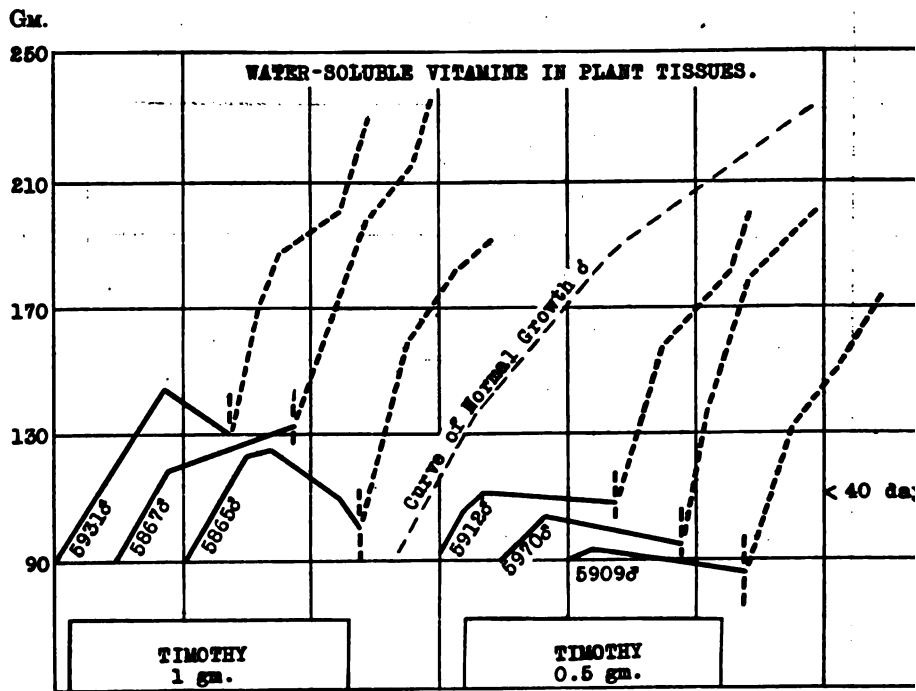


CHART VIII.

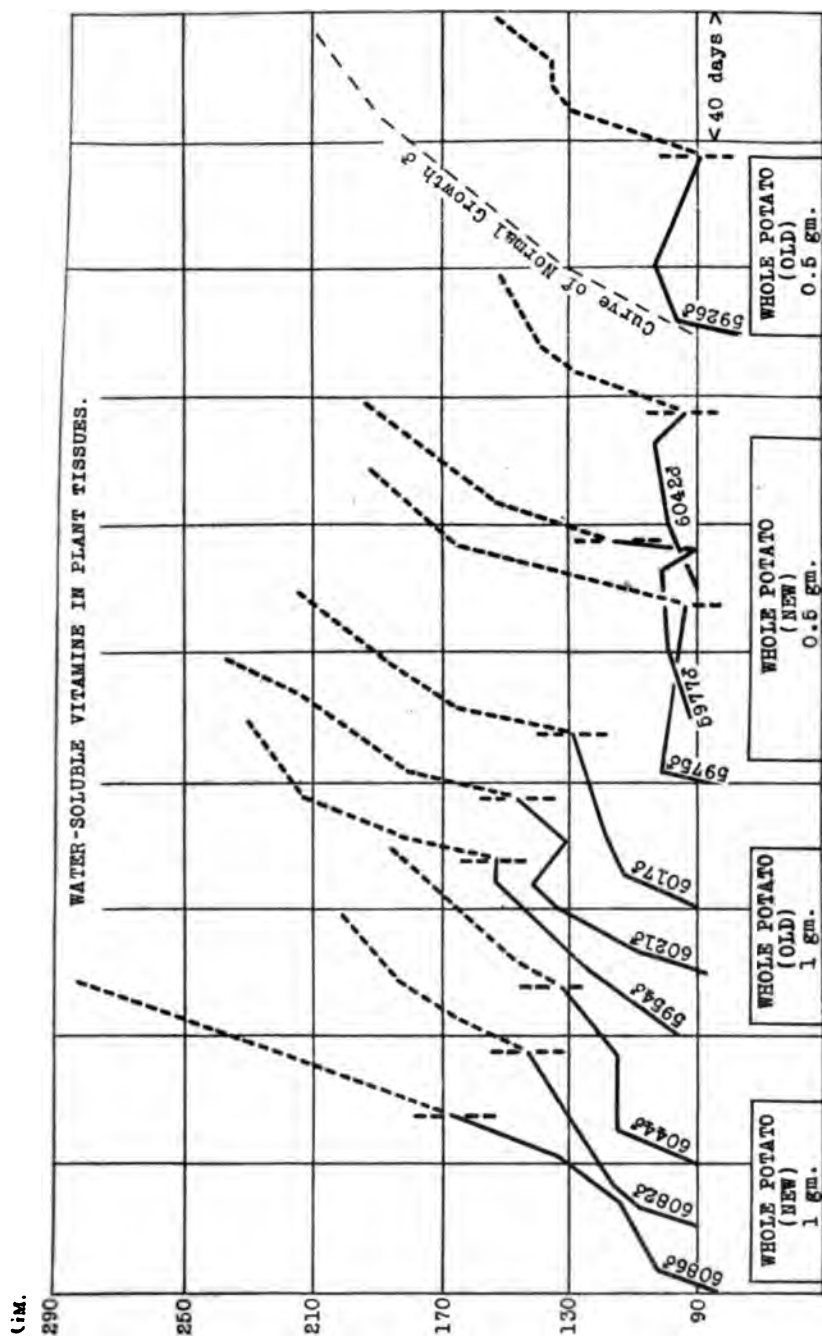


CHART IX.

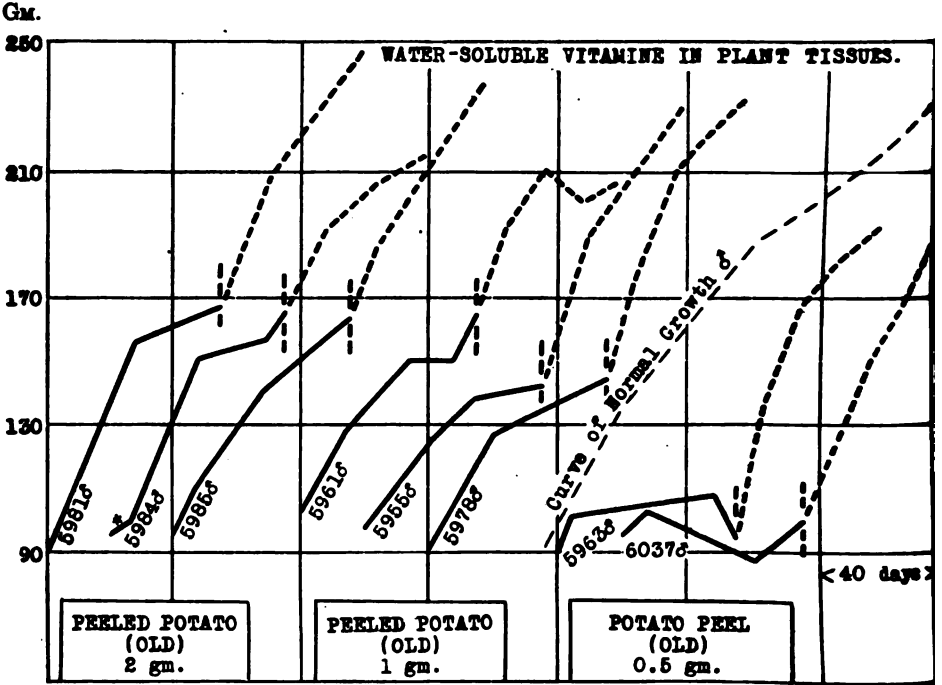


CHART X.

**PROCEEDINGS OF THE AMERICAN SOCIETY OF
BIOLOGICAL CHEMISTS.**

THIRTEENTH ANNUAL MEETING.

Baltimore, Md., April 24-26, 1919.



DETERMINATION OF AMINO NITROGEN IN COMPOUNDS REACTING SLOWLY WITH NITROUS ACID.

By D. WRIGHT WILSON.

(From the Laboratory of Physiological Chemistry, the Johns Hopkins University, Baltimore.)

The rates of reaction of a number of compounds with nitrous acid were studied. Guanine, guanosine, and guanylic acid each give a quantity of gas equivalent to a considerable fraction more than one nitrogen in 1 to 2 hours at 22–25°. Adenine and adenine nucleotide each give exactly the equivalent of one nitrogen. Xanthine and hypoxanthine give off small quantities of gas but uric acid gives none. Cytosine reacts abnormally yielding 137 per cent of the calculated amount of gas in 3 hours. Uracil, thymine, and uridin do not react.

Allantoin reacts slowly and continuously giving off nearly two nitrogens in 5 hours. Substituted amino groups as found in betaine and sarcosine do not react. While creatine fails to give more than traces of gas in 30 minutes, creatinine reacts rapidly yielding gas equivalent to one nitrogen in 1 hour. The action of nitrous acid on creatinine may account for about one-third of the correction for "slowly reacting amines" met with in Van Slyke's determination of amino nitrogen in the urine. The rate of reaction of the various compounds varies considerably with the temperature.

GROWTH EXPERIMENTS WITH PHASEOLIN FROM THE NAVY BEAN.

By C. O. JOHNS, A. J. FINKS, AND MABEL S. PAUL.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

Nutrition experiments with phaseolin, the principal protein of the navy bean (*Phaseolus vulgaris*), show that this protein, after it is treated with dilute alkali and supplemented with 2

per cent of cystine, produces normal growth when it is the sole source of protein in an otherwise adequate diet. Further work is in progress to determine whether alkaline treatment of this protein is necessary for normal growth. The proteins of other beans are being studied in a similar manner.

HYDROLYSIS OF STIZOLOBIN.

By D. BREESE JONES AND CARL O. JOHNS.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

Stizolobin, the principal protein extracted from the Chinese velvet bean, *Stizolobium niveum*, gave the following percentages of amino-acids on hydrolysis with 20 per cent hydrochloric acid: Glycine 1.66, alanine 2.41, valine 2.88, leucine 9.02, proline 4.00, phenylalanine 3.10, aspartic acid 9.23, serine 0.67, tyrosine 6.24, cystine 1.13, arginine 7.14, histidine 2.27, lysine 8.51, ammonia 1.55. Tryptophane was present. Tyrosine was determined both by the colorimetric method of Folin and Denis, and by direct isolation, the latter method giving 5.25 per cent. A method for the direct determination of proline is described, which consists in removing the bases from the hydrolysis solution by means of phosphotungstic acid, extracting the remaining dry, powdered amino-acids with boiling absolute alcohol. Both the total nitrogen and amino nitrogen in the extract are determined and the percentage of proline is calculated from the resulting data.

DETERMINATIONS OF THE HYDROGEN ION CONCENTRATION OF FOODS DURING STORAGE AND PREPARATION IN RELATION TO PRESERVATION OF ANTISCORBUTIC PROPERTIES.

By J. F. McCLENDON AND PAUL F. SHARP.

(From the Physiological Laboratory, University of Minnesota Medical School, Minneapolis.)

The juices of green malt, carrots, cabbage, potatoes, turnips, and lean beef were all found to be distinctly acid whether fresh, or after cold storage, or after boiling in an open vessel. The

meat was first triturated in distilled water. The food was simply placed in a canvas bag, the bag placed in a Buchner press, and the juice pressed out. The juice pressed out of boiled food was acid and the juice boiled after pressing out of fresh food was acid. The unboiled preparations became more acid on standing, but the boiled preparations were so constant as to indicate that their content of volatile acids or bases was very low.

All measurements were made with the hydrogen electrode in which a gold disc was coated with platinum or palladium black. Results with the disc coated with palladium or only partly immersed could not be duplicated. Results with platinum-coated discs entirely immersed were constant and could be duplicated. Apparently the acids attack palladium and the air-surface film has an abnormal hydrogen ion concentration and must not be near the platinum black coating.

Since antiscorbutic vitamins are sensitive to alkalis the results are encouraging. A malt extract intended to cure scurvy was found to be acid during all steps of preparation. Green malt was ground and mashed and at the end of 1 hour momentarily raised to 70°, and, after all the starch was hydrolyzed, condensed *in vacuo* to the point at which bacteria no longer attacked it.

FAILURE OF ACID-FORMING DIET TO CHANGE THE ALKALINE RESERVE OF DOG'S BLOOD.

By J. F. McCLENDON, OSCAR J. ENGSTRAND, AND FRANCES KING.

(From the Physiological Laboratory, University of Minnesota Medical School, Minneapolis.)

By titration in the rotating hydrogen electrode it was found that 0.0295 to 0.0300 cc. of normal HCl was required to neutralize 1 cc. of blood plasma on several normal dogs. A dog was fed on raw lean beef for about 2 months. Blood drawn from a vein at the end of 1 month and from the carotid at the end of 2 months and centrifuged without exposure to air was found to have the same alkaline reserve as the controls. The ash of the food would have required 1,500 cc. of normal KOH for neutralization. We believe that acid-forming diets may be detrimental because they may be deficient in vitamins. Cereals and meat (especially as usually cooked) are deficient in antiscorbutic vitamins. A raw meat diet seems to be sufficient for dogs or men.

RELATIVE LENGTH OF THE INTESTINE IS MORE IMPORTANT
THAN THE CHARACTER OF THE FOOD IN DETERMINING
THE HYDROGEN ION CONCENTRATION OF
INTESTINAL CONTENTS.

By J. F. McCLENDON, LEO C. CULLIGAN, CARL S. GYDESEN,
AND FRANK J. MYERS.

(From the Physiological Laboratory, University of Minnesota Medical
School, Minneapolis.)

Working on the supposition that we attribute to Metchnikoff that flooding the intestine with soluble carbohydrate in the presence of lactic acid bacteria or *Bacillus acidophilus* should cause an increased hydrogen ion concentration of the ileum, we experimented on pups, dogs, cats, and rabbits. We found the ileum content of pups, dogs, and cats acid throughout the entire length and very little changed by changing the soluble carbohydrate (lactose or other sugar) content of the food. The duodenum and proximal part of the ileum of rabbits are acid but the ileum may become slightly alkaline near the cecum. This alkalinity was not prevented by adding lactose to the diet. On averaging the results of Long and Fenger¹ we conclude that the upper third of the ileum of hogs, calves, and lambs is slightly acid and averages 0.215 atmospheres of CO₂ tension and the lower third is neutral or slightly alkaline and averages 0.14 atmospheres of CO₂ pressure. Apparently the CO₂ generated by the action of the gastric contents on the pancreatic juice makes the reaction acid and this acidity is kept up in a short intestine by amino-acids. In a long intestine the CO₂ and amino-acids are largely absorbed before the cecum is reached and the reaction may become alkaline. If acid fermentation of sugars occurs the acids are absorbed so rapidly that they do not prevent the alkaline reaction in the long intestine of herbivora. By forced feeding with sugar a marked diarrhea may be produced and a slightly increased acidity result. The replacement of *Bacillus acidophilus* by *Bacillus coli* in the weening of pups does not result in a marked lowering of the hydrogen ion concentration of the ileum.

¹ Long, J. H., and Fenger, F., *J. Am. Chem. Soc.*, 1917, xxxix, 1278.

FAT-SOLUBLE VITAMINE OF GREEN FOODS.

BY THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL.

(From the Laboratory of the Connecticut Agricultural Experiment Station
and the Sheffield Laboratory of Physiological Chemistry, Yale
University, New Haven.)

Our evidence for the occurrence of fat-soluble vitamine in certain green foods,² which has hitherto been demonstrated only by the use of the food products as a whole in the diet can now be supplemented with experiments in which ether extracts of the plant tissues have proved similarly efficient. McCollum, Simmonds, and Pitz³ have stated that "ether extraction of plant tissues does not remove the substances essential for growth which is contained in butter fat." We have, however, obtained potent preparations as follows: Spinach leaves and young clover respectively, dried in a current of air at about 60°, were extracted with U. S. P. ether. The resultant green extract, yielding an oily residue equal to about 3 per cent of the dried plant, was evaporated upon starch. These preparations, fed in daily quantities equivalent to 1 to 2 gm. of the dried plant, promoted recovery and renewal of growth in rats declining on diets deficient in fat-soluble vitamine. Inasmuch as only 30 mg. per day of the ether extract of spinach sufficed for this purpose it appears that this product ranks among the most potent of the oils heretofore tested. The effects of spinach oil and clover oil in restoring growth in rats that had declined on a diet lacking the fat-soluble vitamine were exhibited in graphic charts of changes in body weight.

ARE THE ANTINEURITIC AND THE WATER-SOLUBLE B VITAMINES THE SAME?

BY A. D. EMMETT AND G. O. LUROS.

(From the Research Laboratory of Parke, Davis and Company, Detroit.)

The chief point in this series of experiments was to study the effect of feeding the same basal food, natural brown rice, to *both* pigeons and young rats, and to determine the effect on the onset

² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187.

³ McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 363.

of polyneuritis and on the rate of growth. The rice was the sole source of the water-soluble vitamine. It was supplemented, for the rats, so that the diet was complete in all other respects. The effect of heat on the water-soluble vitamine in the rice was the criterion for making the distinction.

It was found, upon heating the rice for 2 and 6 hours respectively in the autoclave at 15 pounds pressure, that the antineutritic vitamine was destroyed while the growth-promoting vitamine (water-soluble B) was not so altered. The rate of growth and the food intake varied inversely with the length of time of heating.

We took vitamine extracts of brewer's yeast, and activated fullers' earth vitamine preparations from yeast and protein-free milk, heated these in the same manner as the rice, and tried them out on rats and pigeons. Pigeons could not be cured of polyneuritis nor could the onset of the disease be delayed. Young rats that were suffering from the lack of the water-soluble B immediately began to grow. The possibility of toxic substances in the heated rice having thus been removed, the evidence seemed to be clear that these two vitamines were not one and the same as has been generally claimed. Further study is in progress to enable us to substantiate this hypothesis.

STABILITY OF LACTALBUMIN TOWARDS HEAT.

BY A. D. EMMETT AND G. O. LUROS.

(From the Research Laboratory of Parke, Davis and Company, Detroit.)

Since casein has been claimed to be susceptible to heat and also to be a better protein for growth than lactalbumin, the nutritive value of the latter was studied from the standpoint of its stability toward high temperature. Lactalbumin was heated in an air oven for 2 hours at 120°, and in an autoclave for 1, 2, and 6 hours respectively at 15 pounds pressure, temperature 120°. The heated protein was incorporated in a ration which was complete for normal growth in all respects. Control tests were carried out with lactalbumin that had been dried *in vacuo* at 55-60°. The amount of fat-soluble A (butter fat) was varied also ranging from 5 to 28 per cent.

It was found that heating had practically no effect upon the nutritive value of the lactalbumin even when heated for 6 hours in the autoclave. The higher amounts of butter fat rendered the rations more economical, that is, of the three groups of rats on the 2 hour autoclaved lactalbumin, fed respectively 5, 18, and 28 per cent fat, the 28 per cent fat diet made better gains per gm. of food than did the 5 per cent fat diet. The effect of heat appears to be due to the partial destruction of a water-soluble vitamine other than the growth-promoting accessory, water-soluble B.

NUTRITIONAL STUDIES ON GROWTH OF FROG LARVÆ (*RANA PIPPIENS*).

By A. D. EMMETT AND FLOYD P. ALLEN.

(*From the Research Laboratory of Parke, Davis and Company, Detroit.*)

Tadpoles soon after hatching were grouped and each lot of 500 was fed a definite diet which varied in respect to the kind and amount of protein, the kind and source of water-soluble and fat-soluble vitamins, the kind of carbohydrate, and the amount of fat. Detailed observations were made of the rate of growth (size) and development (length of hind legs). Experimental conditions were maintained exactly the same for all groups including the controls.

It was found that the quality of protein was a much more important factor than the amount; that both vitamins were essential, the water-soluble type being perhaps the more important; that dextrin was no more available than starch; and that the amount of fat present was a very important factor to be borne in mind—large amounts inhibiting both growth and development. In these preliminary studies, it was evident that some factor or factors other than those which were needed for normal growth and development of the rat was essential to the full, vigorous maturity of tadpoles.

DISTRIBUTION OF THE ANTINEURITIC VITAMINE IN THE WHEAT AND CORN KERNEL.

BY CARL VOEGTLIN AND C. N. MYERS.

(From the Division of Pharmacology, Hygienic Laboratory, Washington.)

Feeding experiments on pigeons with corn and wheat, from which the portion containing the embryo had been eliminated, have shown that these foods are practically devoid of antineuritic vitamine. The pigeons developed polyneuritic symptoms after 3 to 4 weeks. Alcoholic extracts of the portion containing the germ, when administered to these polyneuritic pigeons, relieved the symptoms. When changed to an exclusive diet of whole wheat or corn the pigeons also recovered and remained healthy for weeks. It is therefore concluded that (1) the aleurone cells are not the seat of the antineuritic vitamine and (2) that this substance resides in the portion containing the germ and probably within the germ.

COMPARATIVE METABOLISM OF P-NITROPHENYLACETIC ACID.

BY CARL. P. SHERWIN.

(From the Chemical Laboratory of Fordham University Medical School, New York.)

After the ingestion of *p*-nitrophenylacetic acid by a man, only the free acid could be found in the urine and none apparently existed in a conjugated form. 68.70 per cent of the acid fed was extracted from the urine.

After feeding the acid to a dog only 45 per cent of the amount fed was recovered from the urine. Of this amount, 30 per cent was excreted as free *p*-nitrophenylacetic acid while 15 per cent was combined with glycocholic acid and excreted as *p*-nitrophenylacetic acid.

A hen was fed small doses (1 gm. or less) of the *p*-nitrophenylacetic acid. Some of the free acid was extracted from the excreta, but most of the acid was found to be combined with ornithine. This compound on analysis gave results corresponding to the formula $C_{21}H_{22}N_4O_8$.

The acid is soluble in alcohol but insoluble in ether and cold water. The sodium and potassium salts were found to be very hygroscopic and in water solution were dextrorotatory.

The compound was hydrolyzed by boiling with 30 per cent HCl. *p*-Nitrophenylacetic acid was extracted with ether from this acid mixture and identified. The HCl solution remaining was evaporated *in vacuo*. The residue was made alkaline and shaken with benzoyl chloride, then acidified. Crystals of ornithinic acid appeared.

The compound isolated from the excreta of the hen is undoubtedly *p*-nitrophenacetornithinic acid, composed of 1 molecule of ornithine and 2 molecules of *p*-nitrophenylacetic acid.

NATURE OF THE TOXIC AGENT IN MEAT POISONING.

BY ISIDOR GREENWALD.

(*From the Harriman Research Laboratory, the Roosevelt Hospital, New York, in Cooperation with the Section of Food and Nutrition, Medical Department, United States Army.*)

Fresh beef was infected with cultures of *Bacillus paratyphosus* B, *Bacillus enteriditis*, etc. After standing for from 2 to 10 days, the organisms were killed by heating the jar containing the meat to 80° for 30 minutes. The meat was then fed, for from 6 to 8 days, to rats, mice, and guinea pigs. No ill effects were observed. Similar results were obtained with beef and veal condemned because of septicemia, etc. in the animal.

Precipitation by mercuric acetate cannot be employed for the isolation of methylguanidine. The precipitation is not complete and methylguanidine is formed by the oxidation of creatine. Using a method which is free from these objections, no methylguanidine could be isolated from either fresh meat or that which had been allowed to undergo several days bacterial decomposition. Added methylguanidine was recovered, quantitatively.

PRELIMINARY EXPERIMENTS OF THE INFLUENCE OF AMINO-ACID ON THE DIASTATIC HYDROLYSIS OF STARCH.

BY H. C. SHERMAN AND FLORENCE WALKER.

(*From Columbia University, New York, and Carnegie Institution of Washington, Washington.*)

Wheat, maize, and rice starches, similarly purified, showed equal digestibility or rate of diastatic hydrolysis. This was true whether the enzyme employed was purified pancreatic amylase,

commercial pancreatin, saliva, purified malt amylase, malt extract, purified amylase of *Aspergillus oryzae*, or commercial taka-diastrase. With all these except purified pancreatic amylase, potato starch showed a rate of hydrolysis equal to or slightly greater than that observed with the cereal starches. The tendency to abnormally low results in the case noted can be corrected by the addition either of a boiled, carefully neutralized, water extract of potato, or neutralized aspartic acid. Neutralized aspartic acid also increased the rate of transformation of all four of the starches tested, when the enzyme employed was purified pancreatic or malt amylase, commercial pancreatin, or saliva, but did not influence the rate of action of malt extract or taka-diastrase. It was demonstrated electrometrically that the neutralized amino-acid added in these experiments was without effect upon the hydrogen ion concentration of the digestion mixture. The experiments are being continued and extended to other amino-acids and acid amides.

A CORRELATION ON THE OCCURRENCE OF THE FAT-SOLUBLE VITAMINE.

By H. STEENBOCK, P. W. BOUTWELL, AND HAZEL E. KENT.

(From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.)

* In the generalizations sometimes indulged in, when evaluating naturally occurring foodstuffs for specific dietary properties, there has been a tendency to associate a high concentration of the vitamins with the metabolically more active tissues. While this may have been justifiable in certain instances, yet such teleological reasoning may lead to conclusions far from the truth. This has been brought out in experiments carried out to determine the fat-soluble vitamin content of tubers and roots. Presumably all tubers and roots function in the capacity of storage organs for the storage of materials necessary for nursing the growth of the following year's sprouts, yet among them there is a tremendous variation in the fat-soluble vitamin content. This is of such an order that it cannot be correlated with difference in amount of growing tissues. However, there appears to be some relation between yellow pigmentation and vitamin content.

Sweet potatoes and carrots, both highly pigmented, are very rich in the fat-soluble vitamine, while Irish potatoes, mangels, dash-eens, and sugar beets, all carrying little or no pigment, are poor in it. Somewhat similar relations obtain with maize, although it is not as yet justifiable to state it as a general principle. On sound kernels of white maize, suitably supplemented with protein and salts, it has been found impossible to keep young rats alive longer than a few months—death usually resulting after xerophthalmia had set in. On the other hand, on yellow maize, under the same experimental conditions, young rats have grown to maturity at the normal rate, have maintained themselves in excellent condition for months, and have reproduced repeatedly. It is certain that maize, as one of our cereal grains, is by no means always as deficient in the fat-soluble vitamine as present day statements in current contributions would lead one to believe. It is possible that these observations of pigment and vitamine content may give a clue to the manipulations necessary in a procedure leading to the isolation of the fat-soluble vitamine. It is not meant to infer that the vitamine is necessarily a colored compound, but its association with yellow plant pigments is suggestive of its possible chemical nature. It is not carotin.

ON THE PROBLEM OF THE PRODUCTION OF FAT FROM PROTEIN IN THE DOG.

By H. V. ATKINSON AND GRAHAM LUSK.

(From the Physiological Laboratory of Cornell University Medical College,
New York.)

In eight experiments in a series of thirteen, after giving meat in large quantities (700 to 1,300 gm.) to a dog weighing 11 kilos, the respiratory quotients during the height of protein metabolism were between 0.793 and 0.800. A calculation showed that under these conditions the retained carbon residue of the protein metabolized was held back in such a form that, had it been oxidized, the respiratory quotient of this retained pabulum would have been 0.85. This would represent the oxidation of material half of whose calories were composed of fat and half of carbohydrate. The dog showed quotients of 0.82 and above only after the larger quantities of meat were given (1,000 gm. or more). It was

extremely difficult to induce the dog to take meat in sufficient quantity to indicate a considerable production of fat from protein. Incidentally it was observed that the basal metabolism of a dog fed with meat in large quantity for a time and then caused to revert to a standard diet (meat, 100 gm.; lard, 20 gm.; and biscuit meal, 100 gm.) remained persistently (even after 2.5 weeks) at a higher level than had obtained prior to the meat ingestion. This confirms F. G. Benedict's idea of a higher basal metabolism in the presence of "surplus" cellular nitrogen, or the "improvement quota" of protein according to Rubner's terminology.

NOTE ON THE COLORIMETRIC DETERMINATION OF LACTOSE.

By JOSEPH BOCK.

(From the Department of Physiological Chemistry, Marquette School of Medicine, Milwaukee.)

The reaction of lactose with picric acid was studied. The procedure used is similar to the one used by S. R. Benedict in the colorimetric determination of dextrose. The lactose is hydrolyzed before being heated with picric acid and sodium carbonate. The unhydrolyzed lactose gives less uniform results and less intense color development.

The lactose in milk is being determined by this method. After hydrolyzing the lactose, the milk proteins are removed by precipitation with picric acid. An aliquot of the filtrate is used for the lactose determination. 1 cc. or less of milk is used.

Details of the procedure will be published in the near future. The reaction of other carbohydrates with picric acid is being studied.

URINARY OUTPUT OF NITROGEN, CHLORINE, CALCIUM, AND MAGNESIUM IN DIABETES MELLITUS.

By C. FERDINAND NELSON.

(From the Laboratory of Biological Chemistry, University of Kansas, Lawrence.)

Determinations of calcium, magnesium, and sodium chloride in nineteen cases of diabetes mellitus show variations as follows:

Calcium (metal).....	100- 791 mg.
Magnesium "	88-1,049 "
Sodium chloride.....	1.7-15.83 gm.

The total nitrogen varied from 4.58 to 18.40 gm.

Magnesium was excreted in amounts larger than calcium in 26 per cent of the cases studied.

Eleven determinations, made from 5 to 21 days after six of the above cases had been rendered free from sugar, showed a striking change in the urinary calcium and magnesium ratio, magnesium appearing in larger amounts than calcium in 72 per cent of the determinations.

The total nitrogen in each of the latter cases was from 1 to 6 gm. higher than in the former.

CALCIUM AND MAGNESIUM CONTENT OF NORMAL URINE. II.

By C. FERDINAND NELSON.

(From the Laboratory of Biological Chemistry, University of Kansas, Lawrence.)

Additional determinations of the urinary output of calcium and magnesium in normal men and women, on mixed diets in no way restricted or modified except by appetite, show in 58 cases (41 men and 17 women) extreme variations of calcium and magnesium as follows:

		mg.
Calcium (metal)	{ Men.....	416-87
	{ Women.....	265-28
Magnesium "	{ Men.....	305-30
	{ Women.....	166-39

Calcium occurs in larger amounts than magnesium in urine in from 74 to 84 per cent of all determinations made in this laboratory. Magnesium predominates in from 16 to 24 per cent.

Analyses of 3 hour samples of urine, in cases where the magnesium output exceeds that of calcium, show a constant preponderance of the former element throughout the periods observed.

The nitrogen output in cases where magnesium exceeds calcium in the urine is quite as high or may be even higher than where calcium is excreted in the larger amount.

The ingestion of calcium-rich foods, such as milk, promptly increases both the urinary calcium and magnesium outputs.

AVERAGE FOOD CONSUMPTION IN TRAINING CAMPS OF THE UNITED STATES ARMY.

BY JOHN R. MURLIN,

*Lieutenant Colonel, Sanitary Corps, U. S. Army.**(From the Division of Food and Nutrition, Medical Department, United States Army.)*

A comparison was shown of the various rations of the Allied armies, those used in the training period, and those used in active campaign. The average food consumption in 427 messes scattered over 67 different camps including forty-nine divisional and other large concentration camps, fourteen aviation fields, three war prison barracks, one recruiting station, and one spruce production camp is 3,633 calories. The weighted average amounts to 3,625 calories. To this must be added the average food consumption from the canteen or post exchange. Studies in 261 post exchanges in these camps disclose an actual average *per capita* consumption of 365 calories daily. This makes a total food consumption for the average soldier in training of in round numbers 4,000 calories (3,998). Averages by months indicate a well marked, though slight, seasonal variation. The same is shown also when the various messes studied are arranged according to the average prevailing temperature at the time of the surveys.

As compared with the consumption in civilian households the army uses more than twice as much fresh meat, beans, and dried fruit, one and a half times as much bread and bakery products and potatoes, the same amount of sugar, eggs, and lard, much less fresh vegetables, fresh fruits, butter, and milk.

Of the 4,000 calories consumed by the average soldier in heavy training 13 per cent is protein, 31 per cent fat, and 56 per cent carbohydrate. Neglecting the canteen consumption, the distribution is 14 per cent protein, 31 per cent fat, and 55 per cent carbohydrate. As compared with civilian dietaries the percentage of protein is higher, the percentage of fat about the same, and the percentage of carbohydrate a little less.

The average recruit on this diet has gained nearly 7 pounds in a period of 5 months training; the gain is evenly distributed over men of different initial weight.

**VARIATIONS IN STRENGTH AND IN THE CONSUMPTION OF
FOOD BY RECRUITS AND BY SEASONED TROOPS.**

BY PAUL E. HOWE,

*Captain, Sanitary Corps, U. S. Army.**(From the Section of Food and Nutrition, Medical Department, United
States Army.)*

Determinations were made of the variations in strength, using the Martin strength test (by Lieut. C. C. Mason), and weight of approximately forty men from each of eight companies of recruits. The studies extended for 3 consecutive weeks; a smaller number of men were studied for 4 weeks. Weights and strength were determined at 7 day intervals. The food consumption of the companies from which the men were taken was determined (by Lieut. S. C. Dinsmore) in weekly periods. In each period one group of four companies used the same menu and the other four companies a different menu but applicable to all four companies. The men were inoculated and vaccinated during the first 2 weeks of study.

In the first test the men had an average strength which was rather high grade for civilians according to Martin's classification (low B), 757 lbs. Both strength and weight had decreased on the average at the second test: weight, 145.7 to 144.7 lbs., strength, to 705 lbs. The third test indicated that the men had returned approximately to normal: weight, 146.3 lbs.; strength, 743 lbs. The men tested the fourth time maintained their status of the 3rd week or increased slightly in both weight and strength. Conflicting values were obtained with regard to the relation of the proximity of the inoculation to the succeeding strength test. The changes in weight agree with those obtained by Lieut. Perlzweig in another camp.

The average consumption of food for all the companies shows a decrease in the 2nd week from 3,436 to 3,248 calories per man per day; the following week there was an increase to 3,542 calories. One of the two groups of four companies showed a gradual decrease in food consumption while the other showed a regular increase. The food ingestion of different groups of men using the same menu showed considerable variations in quantity and the same group of men have a tendency to vary in the quantity of

food consumed on the basis of the weekly average consumption. These variations are apparently not related to weather changes. The variation in food consumption of seasoned troops, four companies, over a period of 4 weeks likewise showed considerable variation. In each comparative case the men were doing approximately the same work.

**EFFECT OF INTRAVENOUS INJECTION OF PANCREAS EMULSION
UPON THE HYPERGLYCEMIA DUE TO DEPANCREATIZATION.**

By ISRAEL S. KLEINER.

*(From the Department of Physiology and Pharmacology of The Rockefeller
Institute for Medical Research.)*

4 years ago, in the course of another investigation, Kleiner and Meltzer⁴ found that an intravenous infusion of pancreas emulsion into depancreatized dogs temporarily reduced the blood sugar in a marked degree—sometimes to a normal level. The three experiments of that report have now been increased to sixteen, with results which confirm the preliminary statement.

The pancreatic emulsion was prepared as follows: Fresh dog's pancreas was hashed and mixed with three or four times its weight of sterile distilled water. After from 1 to 20 hours in the refrigerator it was strained and squeezed through muslin. The fluid was then mixed with five volumes of sterile 0.9 per cent NaCl solution and was injected slowly during the course of about an hour. The injection of this unfiltered faintly acid or neutral fluid caused a marked reduction of the sugar content of the blood (a loss of 0.09 to 0.20 per cent) in ten experiments, while in six the fall was less pronounced (0.02 to 0.07 per cent). For example, in two experiments the glycemia fell from 0.28 to 0.08 per cent and from 0.33 to 0.17 per cent, respectively. The fall began during the injection and reached its maximum 1 or 2 hours after the injection was ended. There was no dilution of the blood.

A reduction in glycosuria also occurred, but this was produced also by emulsions of other organs. These, however, have not produced the above effect on the blood sugar.

⁴ Kleiner, I. S., and Meltzer, S. J., *Proc. Nat. Acad. Sc.*, 1915, i, 338.

**OXIDATION OF LUCIFERIN AND REDUCTION OF OXYLUCIFERIN
OF LUMINOUS ANIMALS.**

BY E. NEWTON HARVEY.

(From the Physiological Laboratory, Princeton University, Princeton.)

Some luminous animals are known to produce their light by the oxidation of a substance called luciferin in the presence of a second substance called luciferase. Luciferase has many characteristics of an enzyme but is slowly used up in oxidizing large quantities of luciferin. A third substance, photophelein, may assist the luciferin-luciferase reaction. In the absence of luciferase, luciferin oxidizes spontaneously but without light production. The oxidation product may be called oxyluciferin. The reaction $\text{luciferin} \rightleftharpoons \text{oxyluciferin}$ is similar to the reaction $\text{leucomethylene blue} \rightleftharpoons \text{methylene blue}$, as the oxyluciferin can be reduced to luciferin again by Schardinger's enzyme of milk or the reductases of animal tissues. Oxyluciferin can also be reduced by H_2S , nascent hydrogen (from Mg powder and acid), by finely divided palladium, and sodium hypophosphite. Dilute acid favors the reduction and dilute alkali favors the oxidation change. Contrary to the opinion of most investigators no extensive oxidative change appears to occur during luminescence and no carbon dioxide is formed.

**ENZYME STUDIES ON DEHYDRATED AS COMPARED WITH FRESH
VEGETABLES.**

BY K. GEORGE FALK, GRACE MCGUIRE, AND EUGENIA BLOUNT.

(From the Harriman Research Laboratory, the Roosevelt Hospital, New York, in Cooperation with the Section of Food and Nutrition, Medical Department, United States Army.)

Oxidase, peroxidase, catalase, and amylase were determined in fresh and dehydrated potatoes, tomatoes, cabbage, yellow and white turnips, and carrots. In general, the enzyme actions were decreased on dehydration, more so by air blast dehydration than by vacuum dehydration. The effects of hydrogen ion concentrations on the actions were studied. The potato amylase apparently acted differently on the starch occurring naturally in potato juice than it did on Lintner prepared starch.

**THE WORK OF THE HARRIMAN RESEARCH LABORATORY IN
AFFILIATION WITH THE DIVISION OF FOOD AND
NUTRITION, MEDICAL DEPARTMENT,
UNITED STATES ARMY.**

By K. GEORGE FALK.

(From the Harriman Research Laboratory, the Roosevelt Hospital, New York, in Cooperation with the Division of Food and Nutrition, Medical Department, United States Army.)

The work was divided into three parts: study of meat, including chemical study of spoilage⁵ and toxicity studies; development of new process of dehydration applicable to all food products including meat and fish; comparative study of enzymes, proteins, and carbohydrates of fresh vegetables and vegetables dehydrated by different processes.

CHEMICAL CHANGES IN THE BLOOD IN ADVANCED NEPHRITIS.

By VICTOR C. MYERS AND JOHN A. KILLIAN.

(From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.)

As is now well known, the blood in cases of advanced (interstitial) nephritis is characterized by a marked increase in all the compounds which go to make up the non-protein nitrogen. Of the three waste products, uric acid, urea, and creatinine, creatinine appears to be most readily eliminated by the kidney, and is therefore the last one to be retained in nephritis. For this reason and also because of its endogenous origin and very constant formation, the kidney is apparently never able to overcome the handicap of a high creatinine accumulation, thus making creatinine a most valuable prognostic test. Of 85 cases having a creatinine of over 5 mg. per 100 cc. of blood (figures up to 33 mg.) 80 have died, three remain unchanged, while two acute cases have recovered (figures of 5.6 and 6.1 mg.). The question naturally arises whether creatinine may not be responsible for some of the terminal symptoms as a result of its conversion to methylguanidine, a point which has not yet been definitely answered.

⁵ Falk, K. G., Baumann, E. J., and McGuire, G., *J. Biol. Chem.*, 1919, xxxvii, 525. Falk, K. G., and McGuire, G., *J. Biol. Chem.*, 1919, xxxvii, 547.

In connection with convulsive symptoms it may be noted that low figures for Ca may be found; we have observed figures as low as 3 to 4 mg. per 100 cc. of blood.

Judging from the first twenty cases in the above series, in which CO₂ estimations were made, severe acidosis is an invariable accompaniment of advanced nephritis, the CO₂ in twelve instances being low enough to afford an explanation of coma and death.

ELIMINATION OF TARTRATES.

By GEORGE ERIC SIMPSON.

(From the Department of Experimental Medicine, School of Medicine, Yale University, New Haven.)

The urinary elimination of dextro tartaric acid was studied after the sodium salt was given subcutaneously or by stomach tube to cats, dogs, and rabbits. The method used for the determination of tartrate was the Halenke and Moeslinger procedure, which involves the titration of precipitated potassium acid tartrate with standard alkali. This method was found to give consistent results when applied to urine.

After subcutaneous administration, tartrate is eliminated in the urine of all species studied in practically the same degree. Between 70 and 90 per cent of tartrate was found in the urine. When successive subcutaneous injections were given without the development of tolerance the amount of tartrate excreted after each injection decreased until finally none was excreted and the animals died.

When tartrate was given to rabbits or dogs by mouth less was eliminated by the kidneys than after subcutaneous administration. In rabbits an average of 4.9 per cent, in dogs an average of 23.9 per cent, of the tartrate administered by stomach tube was found in the urine. It seems hardly possible that tartrates are oxidized within the body. That part which passes the intestinal wall is for the most part excreted unchanged in the urine.

Calcium administration was not found to delay the excretion of tartrate or alter the degree of elimination by the kidneys. Thus calcium precipitation is probably not a factor in tartrate nephritis.

We were unable to recognize any marked effect of diet on the urinary excretion of tartrates, or to explain, by our study, the marked effect of diet on toxicity reported by Salant and his coworkers.

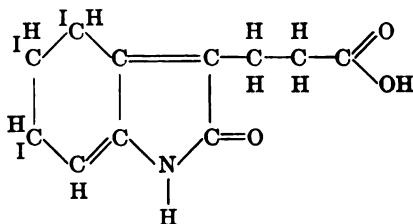
CHEMICAL IDENTIFICATION OF THE THYROID HORMONE.

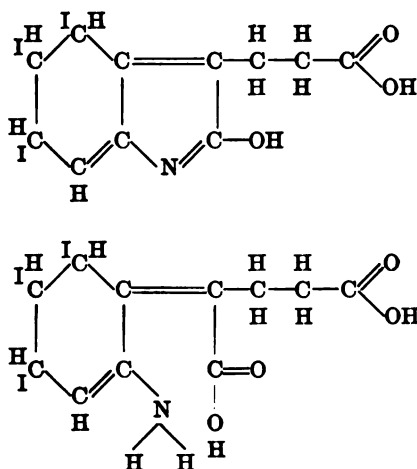
By E. C. KENDALL.

(From the Section of Biochemistry, Mayo Foundation, Rochester.)

The iodine-containing compound which occurs in the thyroid was isolated in crystalline form at the end of the year 1914. Its empirical and structural formulas were determined in May, 1917, and now its synthesis, which has recently been accomplished, completes the chemical work involved in this present investigation. Its chemical structure is related to that of tryptophane, from which it is probably derived. The compound has been named thyro-oxy-indol which has been abbreviated to thyroxin. The data concerning its formula and derivatives were given and the active groups in the molecule CO-NH were discussed. The iodine in the molecule which is attached to the benzene ring does not appear to be involved when the substance affects the energy output, but the chemical groups responsible for its activity are the CO-NH groups which in the body change their form to amino carboxyl groups and the substance, in all probability, functions in this form. The change from CO-NH to amino carboxyl is precisely similar to the opening of the creatinine ring with the formation of creatine. Thyroxin, therefore, although not an α -amino-acid, is essentially an amino-acid and it falls into the well known fundamental groups of substances: amino-acids, protein, creatinine, creatine, etc.

Thyroxin can exist in three forms. The structural formulas are as follows:





DETERMINATION OF ACETONE IN EXPIRED AIR.

By ROGER S. HUBBARD.

(From the Laboratory of Clifton Springs Sanitarium, Clifton Springs.)

A method was described for determining quantitatively acetone in the expired air. Acetone was collected in dilute solution of sodium bisulfite. The subject breathed directly through two bottles containing a 2.5 per cent solution. It was transferred to a Kjeldahl flask and distilled from sulfuric acid and excess potassium permanganate. It was then redistilled from sodium peroxide into Scott-Wilson reagent, and determined by comparing the turbidity with that produced by known amounts of acetone freshly distilled into the same reagent. Solutions of pure acetone added to the breath or to a stream of carbon dioxide gave 90 to 100 per cent recovery.

Normal excretion of acetone per hour as determined by this method ranged from 0.12 to 0.60 mg. and was considerably affected by the diet. Cases showing acidosis gave considerably higher values.

**DIRECT DETERMINATION OF THE NON-AMINO NITROGEN IN
HYDROLYZED PROTEINS.**

BY ALMA HILLER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The former method used by Van Slyke in determining the non-amino nitrogen in hydrolyzed proteins was an indirect one, calculated by subtracting the amino nitrogen from the total nitrogen of the amino-acids. The present method determines this nitrogen directly by employing the following four steps:

1. The removal of phosphotungstic acid from the solution by means of an amyl alcohol-ether mixture, shaking in a separatory funnel, which completely removes the phosphotungstic acid.

2. The removal of the amino nitrogen by means of nitrous acid, in the presence of 10 per cent hydrochloric acid, and by the aid of heat.

3. The removal of the nitrous acid introduced into the reaction by means of reduction with zinc-copper couple.

4. The determination of the remaining or non-amino nitrogen by means of the Kjeldahl method.

**INFLUENCE OF TEMPERATURE ON THE ANTISCORBUTIC
VITAMINE IN TOMATOES.**

BY MAURICE H. GIVENS AND HARRY B. McCLUGGAGE.

(From the Department of Physiology, University of Rochester, Rochester.)

Experiments have been conducted to determine the effect of heat upon the value of tomatoes as an antiscorbutic agent. A daily supplement of 2.5 gm. of fresh raw tomatoes will protect a guinea pig from experimental scurvy when that animal is fed a scurvy-producing diet which is otherwise adequate. If fresh tomatoes are heated at 100°C. for 15 minutes or longer a larger daily dosage must be supplied to insure the animal protection against the disease.

Tomatoes canned according to the usual method of processing, heating under 5 pounds pressure for 10 minutes, will prevent scurvy in a guinea pig when fed in daily portions of 10 gm. This quantity is also sufficient for protection even after heating at 15 pounds pressure for 30 minutes.

Tomatoes dried at 35–40°C. for 32 to 52 hours will protect a guinea pig against experimental scurvy when fed in a daily amount of 0.5 gm. However, this dosage must be increased if the dried tomatoes are cooked before feeding.

The results obtained indicate that the tomato is a very efficient antiscorbutic agent. Further, there is some reduction in its antiscorbutic potency if it is subjected to certain temperature treatments.

THE ISOELECTRIC POINT OF VEGETABLE PROTEIN.

By E. J. COHN.

(From the Harriman Research Laboratory, the Roosevelt Hospital, New York, in Cooperation with the Division of Food and Nutrition, Medical Department, United States Army.)

The chief protein constituent of the potato—tuberin—was studied in connection with an investigation conducted by the Division of Food and Nutrition of the Surgeon General's Office upon the nature of the proteins in certain vegetables. The isoelectric point of tuberin was determined by the method of cataphoresis, and found to coincide with a slightly smaller hydrogen ion concentration than 10^{-4} N. At less acid and at neutral reactions, the protein ionized as anion and migrated to the anode in an electric field. Only at hydrogen ion concentrations greater than 10^{-4} N did the direction of migration change and tuberin ionize as cation.

The effect of sodium chloride upon the solubility of tuberin at different hydrogen ion concentrations illustrates the significance of the isoelectric point. Only at the isoelectric point has sodium chloride no effect upon the solubility of tuberin. At greater acidities, the solubility was decreased by an increase in the concentration of sodium chloride. At neutral reactions sodium chloride increased the solubility of tuberin, which has therefore always been classified as a globulin.

The addition of sodium chloride also has an effect upon the apparent hydrogen potential of otherwise identical systems containing either tuberin and an acid or tuberin and a base. The addition of sodium chloride caused the reaction to converge upon a definite acidity. The addition of salt allows protein to combine

with more acid or more alkali at the same pH everywhere but at the isoelectric point. Previously published data upon gluten claim that gluten exhibits this phenomenon as did tuberin prepared by precipitation at the isoelectric point. Tuberin prepared by dialysis differed in its behavior in that the acidity of systems containing it occasionally converged upon another reaction, characteristic of the method of preparation. Under these circumstances, changes in solubility of the tuberin preparation accompanied those in reaction. These methods of investigation have also been employed in the study of the carrot and the tomato.

COMPOSITION OF BOG BUTTER COMPARED WITH THAT OF ADIPOCERE.

BY R. F. RUTTAN AND L. ISOBEL HOWE.

(From the Department of Chemistry, McGill University, Montreal.)

Eight specimens of bog butter obtained from the Royal Irish Academy of Dublin and seven samples from the Belfast Public Art Museum were analyzed. In Table I will be found the physical and chemical constants of these specimens as compared with those of a very mature sample of pig's adipocere.

The analysis showed:

1. That the hydrolysis of the fats is not so complete in the bog butter as in the adipocere and the percentage of unsaturated fat acids is larger in the bog butter.

2. That the two hydroxy stearic acids which were found by the authors to be present in all samples of adipocere were also found in every sample of the fifteen examined, the average proportion being similar to that found in mature adipocere.

The specimens of bog butter will be seen to vary greatly among themselves in their general composition but like adipocere they have a higher melting point than the original fat and consist essentially of palmitic acid mixed with the two hydroxy stearic acids and a variable but small quantity of oleic acid and fats. Two specimens gave traces of volatile fat acids. Unlike adipocere, soaps were usually absent; traces only were found in one or two specimens.

The theta- and iota-hydroxy stearic acids may be considered characteristic of all "fossil fats."

TABLE I.

Physical and Chemical Constants of Bog Butter Compared with Those of Mature Pig's Adipocere.

	Mature pig's adipocere.	Bog butter.		
		Minimum.	Maximum.	Average of 15 specimens.
Specific gravity (100°C.).....	0.8436			0.8432
Soluble in ether.....	94.1	94.2	98.85	97.25
Melting point, °C.....	60.5	46.0	53.5	49.8
Refractive index at 55°C.....	1.4324	1.4377	1.4463	1.4420
Acid value.....	201.7	153	203.2	173.8
Saponification value.....	270.0	178	218	207.4
Volatile acids, <i>per cent.</i>	None.	None.	0.58	
Hydroxy stearic acids, <i>per cent.</i>	15.80	7.2	25.4	15.21
Neutral fats, <i>per cent.</i>	1.37	6.5	25.8	15.3
Iodine value.....	6.04	7.4	18.2	13.44
Acetyl "	34.75			22.71

THE DETERMINATION OF AMMONIA IN THE BLOOD WITH THE AID OF PERMUTIT.

By S. MORGULIS AND M. JAHR.

TITRATION OF THE BICARBONATE IN BLOOD PLASMA.

By DONALD D. VAN SLYKE AND EDGAR STILLMAN.

DEHYDRATED VEGETABLES FOR ARMY USE.

By S. C. PRESCOTT.

THE ACTION OF ANTAGONISTIC ELECTROLYTES ON THE CONDUCTIVITY OF EMULSIONS AS COMPARED WITH PROTOPLASM.

By G. H. A. CLOWES AND F. WEST.

APPLICATION OF THE PRINCIPLES OF NUTRITION IN AN ARMY CAMP.

By R. J. ANDERSON.

THE ARMY RATION IN FRANCE.

By PHILIP A. SHAFFER.

MILITARY HOSPITAL DIETARIES.

By R. G. HOSKINS.

THE ACID-BASE BALANCE OF FOOD CONSUMED IN ARMY CAMPS.

By N. R. BLATHERWICK.

**A PHYSICO-CHEMICAL METHOD OF CHARACTERIZING
PROTEINS.**

By L. J. HENDERSON AND E. J. COHN.

ON THE CHEMICAL CONSTITUTION OF YEAST NUCLEIC ACID.

By WALTER JONES.

**ARGININE AND ARGINASE IN THEIR RELATION TO THE PRO-
DUCTION OF CREATINURIA.**

By H. STEENBOCK, E. D. GROSS, AND A. KOEHLER.

**THE DETERMINATION OF NITROGEN IN DROP QUANTITIES OF
BLOOD BY DIRECT NESSLERIZATION.**

By AMOS W. PETERS.

**THE FUNCTIONAL CAPACITY OF THE KIDNEYS AND THE BLOOD
FINDINGS IN LATE CASES OF "TRENCH NEPHRITIS."**

By A. P. LOTHROP AND W. T. CONNELL.

**PROCEEDINGS OF THE AMERICAN SOCIETY OF
BIOLOGICAL CHEMISTS.**

FOURTEENTH ANNUAL MEETING.

Cincinnati, Ohio, December 29-31, 1919.



THE ETIOLOGY OF RICKETS.

By E. V. McCOLLUM, NINA SIMMONDS, AND HELEN T. PARSONS.

(From the Laboratory of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

We have conducted an extensive series of experiments with rats restricted to diets derived from cereals and legume seeds; cereals, legume seeds, and muscle meats; and with similar diets in which degerminated products of cereal grains replaced whole seeds; and have supplemented these mixtures with purified food substances to determine the nature and extent of their dietary shortcomings.

In these experiments we have observed the gross picture of rickets in many of the animals restricted to faulty diets, and have demonstrated that this condition develops on diets in which the faults lie in several different factors.

A low content of fat-soluble A, low calcium content, poor quality of protein, and unsatisfactory salt combinations, acting in combinations, may all contribute to the etiology of the disease. We have not yet completed our observations on diets in which but a single factor is at fault. It is certain that specific fasting for fat-soluble A cannot be regarded as the sole and only possible cause of rickets.

Since the same gross picture can be induced in several different ways, we are led to suggest the possible occurrence of more than one kind of rickets. Histological studies of tissues of animals suffering from what appears to be rickets, but from different causes, are still in progress. No decision can yet be reached as to whether in all cases the histological picture is the same in animals exhibiting beaded ribs, enlargements of the costochondral junctions, deformity of the thorax, and general deformity of the body, irrespective of the dietary factor or factors which brought about the condition.

THE RÔLE OF FAT-SOLUBLE VITAMINE IN HUMAN NUTRITION
AND ITS SUGGESTED RELATION TO RICKETS.

By ALFRED F. HESS.

(From the Bureau of Laboratories, Department of Health, New York.)

It has been shown that the fat-soluble vitamine is an essential constituent of the dietary of rats. There have also been clinical reports attributing marked malnutrition in infants and children to a lack of this dietary factor (Japan, Denmark). As a result of these experiences it has been accepted that this vitamine is highly important for man, and that the lack of it leads to nutritional disorder in children. This has been emphasized all the more as this vitamine is not nearly so widely distributed in nature as is the water-soluble vitamine. In order to study this question five infants, varying in age from 5 to 12 months, were given a diet which was complete except for a very small amount of fat-soluble vitamine. It consisted of 180 gm. daily of highly skimmed milk ("Krystalak" 0.2 per cent fat), 30 gm. of cane sugar, 15 to 30 gm. of autolyzed yeast (to supply water-soluble vitamine), 15 cc. of orange juice, 30 gm. of cottonseed oil, and cereal for the older infants.

On this diet the children have done well for a period of 8 to 9 months. They have shown no anemia, no eye trouble, no bone changes, as seen by the x-ray, nor has their growth in length or in weight suffered. We believe, therefore, that either a very small amount of this vitamine suffices to supply the needs of human nutrition, or that this deficiency has to be maintained for a period of years in order to bring about any harmful result. Danger from a lack of this dietary factor need not be apprehended if the diet is otherwise complete.

The development of rickets has been attributed by Mellanby, as a result of experiments on dogs, to a lack of fat-soluble vitamine, and Hopkins and Chick have termed this vitamine the "anti-rachitic factor." It was found, however, that infants fed on this "fat-soluble vitamine minimal diet" did not develop the well established signs of rickets—beading of the ribs, enlargement of the epiphyses, weakness of the muscles, etc. We cannot believe, therefore, that rickets is brought about merely by a deficiency of this principle; all the more so, as this disorder de-

veloped in infants receiving large quantities of milk containing ample fat-soluble vitamine. It may be added that neither cream nor the leafy vegetables, both of which are rich in this principle, are comparable to cod liver oil as growth stimulants.

**PRELIMINARY OBSERVATIONS ON THE RELATION OF BACTERIA
TO EXPERIMENTAL SCURVY IN GUINEA PIGS.**

BY MAURICE H. GIVENS AND GEORGE L. HOFFMANN.

(From the Research Laboratories, Western Pennsylvania Hospital,
Pittsburgh.)

Whether or not bacteria play any rôle in the development of scurvy in guinea pigs has not been settled by direct evidence. Jackson and Moore¹ found coccus-like bodies in microscopic sections of lesions in scorbutic guinea pigs. Jackson and Moody² isolated from the diseased joints, muscles, and lymph glands of these animals Gram-positive and Gram-negative organisms. Pure strains of these bacteria introduced into guinea pigs gave rise in most instances to hemorrhagic and other lesions in the bones, joints, muscles, lymph glands, and organs. Torrey and Hess³ concluded that scurvy, both of guinea pigs and of infants, was not associated with an overgrowth of putrefactive bacteria in the intestinal tract. •

We have attempted to throw further light upon the question by bacteriological examinations of the blood, joints, and feces of guinea pigs made scorbutic on different diets and then treated with different antiscorbutic foods. Blood from scorbutic animals anesthetized and from those dying of the disease regardless of the diet producing the same has been found to be sterile. The enlarged front joints of guinea pigs developing scurvy on oats alone were sterile; this was likewise true in the majority of cases of guinea pigs developing scurvy on the soy cake food of Givens and Cohen.⁴ However, in two or three instances a staphylococcus and diplococcus were isolated. Pure strains of these organisms

¹ Jackson, L., and Moore, J. J., *J. Infect. Dis.*, 1916, xix, 510.

² Jackson, L., and Moody, A. M., *J. Infect. Dis.*, 1916, xix, 511.

³ Torrey, J. C., and Hess, A. F., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 74.

⁴ Givens, M. H., and Cohen, B., *J. Biol. Chem.*, 1918, xxxvi, 127.

injected intracardially, intraperitoneally, and into the joints of healthy guinea pigs on a mixed diet produced no signs of scurvy. Smears and cultures were made of material from different parts of the intestinal tract of guinea pigs on oats alone, on oats plus lemon juice, 3 cc. daily, after scurvy developed, on the soy cake diet, and on the same plus cabbage after the appearance of scurvy. No marked difference was found in the intestinal flora under any of these conditions.

FURTHER STUDIES ON THE USE OF WATER-SOLUBLE B IN THE TREATMENT OF INFANT MALNUTRITION.

By WALTER H. EDDY.

(From the Society of the New York Hospital, New York.)

Results of experiments were reported confirming previous work⁵ of the author in stimulating growth by the addition of B vitamine extract to the diet of infants suffering from malnutrition of the marasmus type. A new feature used in the study was the application of the Bachmann test⁶ to measurement of dosage.

In experiments with vitamine prepared from the navy bean by the McCollum method⁷ the test detected relatively small amounts of vitamine and, while in need of further standardization, offered a valuable aid in measurement of the vitamine B present in the substances used. Tables were shown giving the result of the test on various amounts of the dextrin-vitamine mixture and on other substances such as milk, both cow and human milk.

The first case, showing stimulation with the B vitamine, gained an average of 0.84 ounces per day in a 32 day period as against a gain of 0.47 ounces per day during a 17 day period preceding the use of the vitamine through the calorie intake and the food given remained constant through both periods. The second case showed a similar stimulation though not so well controlled as the first. The interesting feature of the use of the Bachmann test as applied to the first case was the result of the tests as applied to the child's diet and to the extract. The diet

⁵ Eddy, W. H., and Roper, J. C., *Am. J. Dis. Child.*, 1917, xiv, 189.

⁶ Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

⁷ McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

was found to contain 2,120 units of vitamine and the stimulating mixture only 70 units.

In other words an increase of only 3 per cent in actual vitamine intake produced the marked stimulation. The author suggested that this result may be due to the fact that the child could utilize the extracted vitamine when it could not utilize the vitamine in the diet and that the way the vitamine is held in a diet may be an important factor. In all the baby cases treated the extract feeding is followed by an increased growth which continued to a point where removal of the extract is possible without marked reduction in the growth rate and the child then goes on to recovery. These cases represent the fifteenth and sixteenth showing stimulation under this treatment.

THE EXOGENOUS AND ENDOGENOUS NATURE OF THE CREATINURIA IN THE GROWING DOG.

By VICTOR JOHN HARDING AND ELRID G. YOUNG.

(From the Biochemical Laboratory, McGill University, Montreal.)

In investigating the creatinuria of pups, and studying the effect of high and low protein diets upon that condition, the authors find in most of the animals that the creatine excretion is dependent upon the level of protein intake. Only in one animal, however, did they find that the creatinuria could be totally abolished by a low protein diet, and even in this case creatine again made its appearance in the urine in spite of the low protein diet. In most of the animals investigated there always remained a residuum of creatine in the urine, which invariably increased on a continuation of the low protein diet. This increase in the creatine excretion, following its lowering with a low protein diet, is ascribed to the production of that substance from some store of intermediary metabolites. The authors are thus of the opinion that creatine can be of both exogenous and endogenous origin and that both can exist in the same animal. The endogenous creatine is not a constant quantity, and its amount is determined by factors as yet unknown. In two animals, however, the authors were unable to affect the excretion of creatine by an alteration in the protein of the diet. In these animals apparently the endogenous fraction is so large as to obscure the exogenous portion.

CYSTINE AS THE PRECURSOR OF CREATINE IN THE GROWING DOG.

BY VICTOR JOHN HARDING AND ELRID G. YOUNG.

(From the Biochemical Laboratory, McGill University, Montreal.)

The authors have investigated the effects upon the exogenous creatine excretion in pups of variations in the amino-acid content of the protein.

Arginine is without any effect. A variation in the content of the cystine in the protein or the addition and withdrawal of that amino-acid as such is followed by a variation in the creatine excretion. It is found, however, that as well as affecting the exogenous function of the creatine the endogenous portion is also disturbed.

The hypothesis has been formulated that cystine forms creatine through the intermediate stages of taurine and amino ethyl alcohol, followed by methylation, combination with urea, and oxidation.

THE ORIGIN OF CREATINE.

BY R. B. GIBSON AND FRANCES T. MARTIN.

(From the Chemical Research Laboratory, Department of Theory and Practice of Medicine, the State University of Iowa, Iowa City.)

Ingested creatine is promptly eliminated chiefly as creatine, in part as creatinine, in cases of advanced progressive pseudohypertrophic muscular dystrophy. The creatine and to a lesser extent the creatinine are increased with a high protein intake inasmuch as in the case reported half again as much creatinine and twice as much creatine may be excreted as on a low nitrogen plane. This increase is obtained from the catabolized protein (including gelatin) and not from that retained for growth purposes. The substitution of the arginine-rich protein edestin for the protein of the diet does not affect the creatine output. Ingested asparagine and sarcosine do not lead to increased creatine formation; glycocyamine is converted in part (at least 36 per cent) in a controlled experiment, thus confirming earlier work. It is probably not a stage in the ordinary creatine formation.

**CORRELATION OF CERTAIN PHYSICAL AND CHEMICAL FACTORS
WITH TOXICITY TO MARINE ORGANISMS.**

By G. H. A. CLOWES AND L. G. KEITH.

*(From the Biochemical Research Laboratory, Eli Lilly and Company,
Indianapolis.)*

The absolute and relative toxicities of the symmetrical and asymmetrical dichloroacetones to developing sea urchin eggs correspond with their toxicities to mice, the symmetrical compound being sixty times as toxic as the asymmetrical.

Experiments with mustard gas and other war gases have indicated that their relative toxicities depend upon rate of hydrolysis, lipid water distribution coefficient, vapor tension, etc. The dichloroacetone experiments support this view. The symmetrical compound diffuses more rapidly from a non-aqueous to an aqueous phase, and hydrolyzes more rapidly in a feebly alkaline aqueous phase than does the asymmetrical.

With both substances a latent period was observed, during which the gases could be removed by shaking with additional eggs or charcoal, the protective effect depending upon temperature, time of application, and amount of protective substance employed.

An analysis of the data obtained by exposing varying numbers of eggs to varying concentrations for varying times at varying temperatures, and subjecting them to varying protective procedures, supports the view previously advanced that the war gases are first adsorbed on the surface of the protoplasmic structure, then penetrate through protoplasm by diffusion, and finally after a latent period undergo hydrolysis causing the death of the cell.

METABOLISM OF O-NITROBENZALDEHYDE, M-NITROBENZALDEHYDE, AND P-NITROPHENYLACETALDEHYDE.

By CARL P. SHERWIN, JOHN A. DALY, AND WALTER A. HYNES.

(From the Research Laboratory, Fordham University, New York.)

According to the investigations of Cohn, *o*-nitrobenzaldehyde, when fed to the rabbit, is mostly destroyed, and only about 10 per cent. is excreted in the urine as *o*-nitrobenzoic acid. *m*-Ni-

trobenzaldehyde is excreted by the rabbit as *m*-acetylaminobenzoic acid, and by the dog as *m*-nitrohippuric acid urea. *p*-Nitrobenzaldehyde is excreted by the rabbit as a combination of *p*-nitrobenzoic acid and *p*-acetylaminobenzoic acid, and by the dog *p*-nitrobenzaldehyde is excreted in the urine as *p*-nitrohippuric acid urea.

We fed 2 gm. of *o*-nitrobenzaldehyde to a man and recovered 65 to 70 per cent of *o*-nitrobenzoic acid from the urine. No reduction of the nitro group was apparent in this case, nor was there any combination with glycocoll by the *o*-nitrobenzoic acid thus produced.

m-Nitrobenzaldehyde was fed to a man in 5 and 6 gm. doses. From the urine we recovered about 75 per cent of the substance in the form of *m*-nitrobenzoic acid, and after the 6 gm. dose a fraction of a gm., about 5 per cent, was found as *m*-nitrohippuric acid.

p-Nitrophenylacetaldehyde was fed to a rabbit in 1 gm. doses, and 75 to 80 per cent was excreted as *p*-nitrophenylacetic acid, while *p*-nitrophenylacetic acid, when fed, resulted in rapid elimination of the same substance in the urine. 5 gm. of *p*-nitrophenylacetaldehyde were also fed to a dog and excreted in the urine as *p*-nitrophenylacetic acid. *p*-Nitrophenylacetic acid, as previously shown by one of us, is excreted mostly as the same substance in the urine of the dog, but a small fraction was also excreted in combination with glycocoll as *p*-nitrophenaceturic acid. 5 gm. of *p*-nitrophenylacetaldehyde were ingested by a man and 70 per cent of this was recovered from the urine as *p*-nitrophenylacetic acid. *p*-Nitrophenylacetic acid, as we have previously shown, is excreted by the human uncombined.

In these cases we could find no reduction of the nitro group in any of the aldehydes, as found by Cohn in his work on rabbits, so we fed 5 gm. of *p*-aminophenylacetic acid to a human, and again to a dog. In both cases there appeared in the urine a dark red substance, easily soluble in all organic solvents and insoluble in water. This substance has not as yet been crystallized or identified.

**RELATION BETWEEN AMMONIA EXCRETION AND THE
HYDROGEN ION CONCENTRATION OF URINE.**

BY CYRUS H. FISKE.

(From the Biochemical Laboratory, Harvard Medical School, Boston.)

Hasselbach's supposed constant relation between the ammonia coefficient and the C_H of urine is subject to deviations of sufficient frequency to suggest that other controlling factors are concerned. An examination has consequently been made, on hourly samples of human urine, of (1) the C_H , and (2) a ratio, that is more likely than the ammonia coefficient to have a real significance in connection with the regulation of reaction; viz., the ratio of ammonia to sulfuric acid. Other things being equal, this ratio rises with the C_H , but the relation is by no means constant under all conditions. At the same C_H , the ratio is lower the higher the sulfate content, and also the higher the phosphate content. The dependence on the sulfate excretion may be explained by the fact that acids, other than sulfuric, that call for the formation of ammonia, are relatively less in amount the greater the rate of production of sulfuric acid, while the dependence on the phosphate output is perhaps to be accounted for by the supposition that the demand for ammonia is less the greater the amount of phosphate available for excretion, since the conversion of secondary to primary phosphate liberates fixed alkali.

**THE AMINO-ACID CONTENT OF BLOOD FROM THE JUGULAR
AND MAMMARY VEINS OF MILKING COWS.**

BY C. A. CARY.

(From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture.)

An attempt was made to determine the change in the amino-acid content of blood and of blood plasma as it passes through the mammary gland of milking cows by comparing the amino-acid N in samples obtained as nearly simultaneously and with as little disturbance as practical from the jugular and mammary (abdominal subcutaneous) veins.

The proteins were removed by the coagulation-trichloroacetic acid-kaolin method described by Bock,⁸ the amino N being determined by the HNO₂ method, using the smaller apparatus of Van Slyke. The urea, etc. were corrected for by making duplicate runs allowing a longer time for deamination.⁹

The averages of duplicate analyses of plasma are given in Table I.

TABLE I.

Vein.	Amino-acid content of 100 cc. of blood plasma.					
	I	II	III	IV	V	VI
	mg.	mg.	mg.	mg.	mg.	mg.
Jugular	2.68	3.31	2.41	2.49	2.38	2.47
Mammary	1.78	2.34	2.45	1.93	2.00	1.87
Difference	0.90	0.97	0.04	0.56	0.38	0.60

The cows gave about 10 kilos of milk daily. The plasma constituted about 67 per cent by volume of the whole blood. Using a very rough approximation¹⁰ of the flow of blood through the mammary gland, assuming that the difference in amino-acid content of the mixed arterial and jugular bloods is relatively negligible, and that the changes in the plasma thus indicated are effected continuously throughout the 24 hours, our largest differences indicate that about 35 gm. of amino-acid N are abstracted daily from the plasma passing through the gland. This estimate is rough and it is very doubtful whether we get samples of blood indicating the maximum changes occurring in the gland. It is, however, suggestive relative to the formation of milk proteins.

With the whole blood the differences are in general in the same direction as those found in the corresponding plasma, but more work must be done to determine whether there is any change or not in the amino-acid N of the corpuscles of blood perfusing the gland. The work is still in progress.

⁸ Bock, J. C., *J. Biol. Chem.*, 1916-17, xxviii, 357.

⁹ Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

¹⁰ Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 21.

NEUTRALITY REGULATION IN CATTLE.

By N. R. BLATHERWICK.

(From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture.)

The alkaline reserve of the plasma of cows, as measured by the CO₂ capacity, is remarkably constant. The average normal value, 60.9 cc. of CO₂ per 100 cc. of plasma, is somewhat lower than that of normal human plasma. Maximum and minimum values were 65.5 and 55.1 cc., respectively. The CO₂ capacity of the plasma of three pregnant cows showed no evidence of the acidosis of pregnancy observed by others in women. By feeding a cow on rations restricted to one food, namely hay, grain, or silage, changes in plasma CO₂ capacity and in the ammonia and CO₂ of the urine were produced. A cow fasted for 7 days failed to show a decrease in the CO₂ capacity of the plasma, but rather a slight increase apparent at 24 hours, which was maintained throughout the fast. Coincident with this was an increase of 11 per cent in the inorganic P of the plasma. Larger increases in the inorganic P of the plasma, as the result of fasting, have been found in this laboratory. This change indicates a mobilization of bone substance to assist in the maintenance of neutrality. Another important factor in maintaining the correct reaction of the cow's body is the excretion of CO₂ in the urine. This has been observed to vary from 6 cc. per 100 cc. in the urine of a young calf living on a milk diet to 394 cc. in that of a cow eating silage, grain, and hay.

EFFECT OF ACIDS, ALKALIES, AND SALTS UPON CATALASE PRODUCTION.

By W. E. BURGE.

(From the Department of Physiology, University of Illinois, Urbana.)

100 cc. of 0.15 per cent hydrochloric acid decrease the catalase of the blood about 30 per cent in 2 hours, while a similar quantity of acetic acid increases it about 14 per cent. The decrease produced by the hydrochloric acid is due in part to the destruction of the enzyme and in part to the inhibiting action of the acid; the increase produced by the acetic acid is due to the stimulation

of the alimentary glands, particularly the liver, to an increased output of the enzyme. Carbonic acid was found to have no effect on the catalase of the blood. 10 gm. per kilo of sodium carbonate as well as sodium acetate dissolved in 100 cc. of water increase the catalase of the blood. The animals used in these experiments were rabbits and the substances were introduced by means of a stomach tube.

A STUDY OF THE DISTRIBUTION OF CATALASE IN THE KIDNEY.

BY SERGIUS MORGULIS AND VICTOR E. LEVINE.

(From the Department of Biochemistry and Physiology, College of Medicine, Creighton University, Omaha.)

Experiments with the kidney of rabbit, sheep, pig, and beef, perfused with saline and unperfused, indicate a catalase content (quantity of hydrogen peroxide decomposed) and a catalytic activity (rate of decomposition) which are much greater in the cortex than in the medulla. The upper portion of the medulla yields greater values than the papillary portion. Part of the catalase is derived from the blood and can be removed by perfusion; part remains insoluble even after prolonged perfusion. Results with the right and the left kidney from the same animal point to the fact that the two organs differ in catalase content and catalytic activity. The kidney tissue contains no direct oxidizing enzyme. Quantitative experiments on peroxidase show that the peroxidase relations are the opposite of those of catalase. The greatest peroxidase content and peroxidase activity (rate of reaction) are found in the medulla; the cortex values are comparatively small. Perfusing the kidney results in the complete removal of peroxidase, although catalase is removed only in part by this procedure.

**THE SIGNIFICANCE OF CONCENTRATION WITH REFERENCE TO
SUBSTANCES IN THE BLOOD PLASMA.**

By R. T. WOODYATT.

*From the Laboratory of Clinical Research, Rush Medical College, the Otto
S. A. Sprague Memorial Institute, Chicago.)*

A dog weighing 10 kilos received glucose by vein constantly for 8 hours at the uniform rate of 10 gm. per hour. In the first 4 hours the glucose was given in dilute solution; in the second 4 hours in concentrated solution; *i.e.*, first in about 3 and later in about 36 per cent form. The dog passed in the urine 0.1 to 0.2 gm. of sugar per hour throughout (except in the first hour). In the 2nd, 3rd, 4th, and 5th hours the blood sugar percentage was 0.13 to 0.14 per cent. In the 6th, 7th, and 8th hours it was 0.21 to 0.23 per cent. This change followed the change in the volume of water injected and occurred while the rates of glucose injection, glucose elimination, and hence glucose utilization all remained constant. If anything, the glucose excretion was a trifle higher during the period of low blood sugar percentages. The experiment is one of a group and depicts a type.

The phenomenon was discussed in relation to the observations of Magnus on salts; and Epstein on the blood sugar and rate of glycosuria in diabetes. It was pointed out that if the surface of contact between the blood and the cells should vary in direct proportion to the blood volume, the results would be explained. Anatomical details of capillary systems were discussed to show how an increasing volume of capillary blood—especially by opening up erstwhile empty capillary collaterals—may develop surface approximately in proportion to the volume of extra blood.

In interpreting the physiological effects of a substance in the blood plasma, the factor of concentration alone is not sufficient. Surface is also important. Possibly during normal variations of the blood volume, surface and volume vary in direct proportion.

ALKALOID DIFFUSION IN PHYSICAL AND BIOLOGICAL SYSTEMS.

BY G. H. A. CLOWES AND A. L. WALTERS.

*(From the Biochemical Research Laboratory, Eli Lilly and Company,
Indianapolis.)*

Alkaloids adsorbed by fullers' earth cannot be released by extraction with water or an aqueous solution of sodium bicarbonate having the alkalinity of intestinal contents. Nevertheless when the alkaloid adsorption compound is administered by mouth, the alkaloid is adsorbed by the intestinal mucosa. It was found, for example, that adsorbed atropine administered to cats by mouth produced a prolonged dilatation of the pupil of the eye. Adsorbed strychnine caused convulsions and death in rabbits and rats, and adsorbed quinine and emetine were recoverable in the urine. Adsorbed strychnine introduced into the peritoneal cavity caused convulsions and death, but when the adsorbed strychnine was first introduced into a collodion sac which was freely permeable for strychnine but would not permit of the passage of cells, and the sac introduced into the peritoneal cavity, there was no effect.

These experiments show that the alkaloid may be extracted by protoplasm in a neutral medium and suggest the probability that the alkaloid is released by direct contact between the protoplasmic material of the cell and the adsorption compound.

In the above experiments from two to ten times the amount of adsorbed alkaloid was required to produce a result equivalent to that obtained in control experiments with free alkaloid. Alkaloids were extracted from their adsorption compounds to a slight extent by intestinal contents, somewhat better by means of intestinal mucosa, egg yolk, etc., but with considerable facility by so called neutral soaps and soaps to which either fatty acid or alkali had been added. The extracts were carried out either in aqueous or mixed aqueous organic media or in emulsions using soaps of sodium, potassium, calcium, and magnesium.

These results support the view that soaps and the fatty acid group play an important rôle in protoplasmic assimilation, and explain the therapeutic effects obtained by administering alkaloids in the adsorbed form.

**THE NATURE OF THE LIGHT-PRODUCING REACTION OF
LUMINOUS ANIMALS.**

BY E. NEWTON HARVEY.

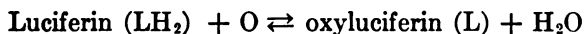
(From the Physiological Laboratory, Princeton University, Princeton.)

Animal luminescence results from the oxidation of a substance, luciferin (LH_2), in presence of a catalyst, luciferase, water, and oxygen. When solutions of luciferin and luciferase are mixed, not enough CO_2 is produced to saturate the buffers in these solutions and probably no CO_2 at all is produced; not enough heat is produced to raise the temperature of the mixture 0.001°C ., and from this figure the heat of oxidation of luciferin is calculated to be less than 0.1 calorie per gm. The oxidation product of luciferin, oxyluciferin (L), is very similar to luciferin and may be reduced to luciferin again by various perhydridases and by nascent hydrogen or H_2S . The reduction is accelerated by acid and in the presence of light. Thus the reaction is photogenic in one direction, photochemical in the opposite direction, and may be represented as follows:

Darkness.

Alkali.

Luciferase.



Perhydridase or nascent H.

Acid.

Light.

**THE INFLUENCE OF OXYGEN DEFICIENCY AND RELATED
CONDITIONS UPON THE HEMATO-RESPIRATORY
FUNCTIONS.**

BY YANDELL HENDERSON AND HOWARD W. HAGGARD.

*(From the Physiological Laboratory, School of Medicine, Yale University,
New Haven.)*

It is generally supposed that under oxygen deficiency acids are produced in the tissues and are retained in the blood, and that the blood alkali is thus neutralized and eliminated through the urine. This may be termed the acidotic process.

Experiments on dogs subjected to progressively decreasing oxygen show, however, that the process actually involved is in many respects exactly the opposite of the usual supposition. They demonstrate that before any considerable amount of alkali is lost an abnormally large amount of CO_2 is eliminated by the excessive breathing induced by a lowered oxygen pressure in the air breathed. Then alkali passes out of the blood to compensate this alkalosis. This we term the acapnial process.

From these facts and related observations on men it appears that in normal persons the blood alkali is controlled by the dissolved CO_2 —more or less alkali being called into use in the blood to satisfy the normal $\text{H}_2\text{CO}_3 : \text{NaHCO}_3$ relation, and thus to keep the C_{H} of the blood nearly constant. The amount of dissolved CO_2 in the blood is controlled by the pulmonary ventilation, and fundamentally in normal persons the ventilation is adjusted to the oxygen partial pressure of the altitude at which the person lives.

When, however, overbreathing is induced and the H_2CO_3 of the blood is reduced, the NaHCO_3 follows downward. This, we find, and not acidosis, is what occurs also in carbon monoxide asphyxia.

A simple test for differentiating between low blood alkali of acidotic and that of acapnial origin is the administration of air to which 8 to 10 per cent of CO_2 has been added. This quickly causes death in acidotic subjects; but it induces a rapid recall of alkali to the blood, and restoration of the subject to a virtually normal condition of health if the condition is of acapnial origin.

We have now (in collaboration with Dr. R. C. Coburn) applied this treatment to patients after surgical operation and anesthesia. The result has been that alkali was recalled to the blood, that arterial pressure and other functions were restored to normal, and that the anesthetic was rapidly eliminated by the full breathing during the CO_2 inhalation with a consequent great reduction of nausea.

In dogs asphyxiated with carbon monoxide (in investigations for the United States Bureau of Mines) strikingly beneficial results have been obtained by means of oxygen reenforced with 10 per cent CO_2 .

RELATION OF ANESTHESIA TO RESPIRATION.

By SHIRO TASHIRO.

(From the Biochemical Laboratory, College of Medicine, University of Cincinnati, Cincinnati.)

The rate of the tissue respiration of the claw nerves of various crabs is highest in lobster, then spider crab, and least in *Limulus*. That of the sciatic nerve of frog comes between the last two. The time required for complete anesthesia with the same narcotics is shortest for lobster, then spider crab, frog, and *Limulus* in order. Susceptibility of the nerve to anesthetics runs parallel to the rate of its respiration, provided that other factors are fairly constant. A 0.4 per cent solution of chloral hydrate does not alter the original gradient of respiration of the spider crab's nerve, but 2 per cent, which produces reversible loss of irritability, reverses it. These results can be best explained if we accept the idea that susceptibility of the same nerve toward anesthetics is quite different along the fiber, depending mainly upon the different respiratory activities of the different parts of the fiber. The advantage of using tissue with as few functions as possible is obvious, for we must know exact physiological conditions of all parts of the tissue when we measure the respiration. Abolition of one function in a tissue may or may not mean complete anesthesia of the tissue as a whole. It is suggested that primary stimulation by weak concentrations of narcotics is probably caused by exaggerating the original metabolic gradient; and final anesthesia, by further oxidative interference which primarily changes the relation between tissue respirations of the different parts of the tissue.

DETERMINATION OF METHEMOGLOBIN IN BLOOD.

By W. S. McELLROY.

(From the Laboratory of Physiological Chemistry, School of Medicine, University of Pittsburgh, Pittsburgh.)

When methemoglobin is present the colorimetric determination of hemoglobin cannot be used as an index of the oxygen-carrying capacity of the blood. Under these conditions the oxygen capacity must be determined directly.

Methemoglobin interferes with the colorimetric determination of hemoglobin as carbon monoxide hemoglobin and acid hematin.

The total hemoglobin in the presence of methemoglobin can be determined colorimetrically by converting the oxyhemoglobin into methemoglobin by means of potassium ferricyanide or other suitable reagent and determining the total as methemoglobin.

The oxygen capacity gives the amount of oxyhemoglobin.

The difference between the total hemoglobin determined as methemoglobin and the oxyhemoglobin estimated from the oxygen capacity gives the amount of methemoglobin.

BLOOD PHOSPHATES IN THE LIPEMIA PRODUCED BY SEVERE HEMORRHAGE.

BY W. R. BLOOR AND E. D. FARRINGTON.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

Changes directly connected with the lipemia were confined to increases in lipid phosphorus in plasma (up to five times the normal value) and in corpuscles (double the normal value).

Changes attributable to the bleeding and regeneration were mainly increases in the inorganic phosphates in the corpuscles and, to a less extent, changes in the quantities of the other phosphoric acid combinations.

FAT CONTENT OF EMBRYONIC LIVERS.

BY C. G. IMRIE AND S. G. GRAHAM.

(From the Laboratory of Pathological Chemistry, University of Toronto, Toronto.)

Observations were made upon the fat content of the livers of embryonic guinea pigs throughout the period of gestation. Such changes, if any, had first to be established before the relation of the embryonic liver to fat mobilized from the storehouses in the pregnant animal could be interpreted. The results of this preliminary investigation were reported. The higher fatty acid content was determined by saponification and extraction, as described by Leathes, and the iodine value by the method of Wij. The weight of the embryo was employed as an indication

of its age. Until the embryo reaches a stage in its development corresponding to a weight of 35 to 40 gm., the fat content of the liver more or less closely approximates that of the maternal liver, which is from 2 to 3 per cent. After this period, however, there is a progressive accumulation of fat in the embryonic liver, so that at birth the animal has from 16 to 18 per cent of higher fatty acids reckoned upon the moist tissue. The iodine value of this fat was comparatively high, though lower than that in the maternal liver.

A study of the fate of this fat showed that it is rapidly utilized by the animal during the first 48 to 72 hours of its life. As the fat content is lowered during this period, the iodine value rises.

Figures and charts representing these changes were presented.

QUANTITATIVE COLORIMETRIC DETERMINATION OF TYROSINE AND TYRAMINE (P-HYDROXYPHENYLETHYLAMINE) AND OTHER PHENOLS.

By MILTON T. HANKE AND KARL K. KOESSLER.

*(From the Otho S. A. Sprague Memorial Institute and the Departments of
Pathology and Physiological Chemistry, University of Chicago,
Chicago.)*

A method has been developed for estimating tyrosine, tyramine *p*-cresol, oxyphenylacetic acid, and phenol by means of which quantities as small as 0.000005 gm. of these substances can be determined. The method is based upon the interaction, in alkaline solution, of the phenols with *p*-phenyldiazonium sulfonate. The color so obtained is intensified and stabilized by the addition of NaOH and hydroxylamine hydrochloride. The theory of the reaction involving the stabilization of the quinone ring by the hydroxylamine was discussed in detail.

**QUANTITATIVE COLORIMETRIC DETERMINATION OF HISTIDINE
AND HISTAMINE IN PROTEINS AND PROTEIN-CONTAINING
MATTER.**

BY KARL K. KOESSLER AND MILTON T. HANKE.

*(From the Otho S. A. Sprague Memorial Institute and the Departments of
Pathology and Physiological Chemistry, University of Chicago,
Chicago.)*

A method has been developed for estimating histidine and histamine in proteins and protein-containing matter that is based upon the method developed for pure imidazoles and previously described by us,¹¹ and upon the method developed by Van Slyke for the estimation of the hexone bases.¹²

DIGESTIBILITY OF RAW CORN-STARCH.

BY C. F. LANGWORTHY AND HARRY J. DEUEL, JR.

*(From the Office of Home Economics, States Relation Service, United States
Department of Agriculture.)*

Raw starches are generally considered to be digested only slightly, if at all, by the human body. In the experiments here reported the digestibility of raw corn-starch when eaten in quantity by normal men was determined. There were some variations, but on an average the three subjects ate 250 gm. of raw corn-starch a day during the 3 day experimental period, in the form of a frozen custard, in which the starch was combined with milk, sugar, and a little salt, and flavored with lemon extract to mask the "starchy" taste. The methods were those usually followed in experiments with cereals carried on in this laboratory. The total amount of feces pertaining to the experimental period was very small and on examination did not show any starch.

The digestibility of the raw corn-starch was found to be 100 per cent. So far as could be seen, its ingestion had no abnormal physiological effects and the subjects remained in their accustomed good health.

The experiments here reported are the first of a series dealing with the digestibility of raw starches, in which it is planned to include potato, rice, wheat, arrowroot, and possibly other starches.

¹¹ Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 521.

¹² Van Slyke, D. D., *J. Biol. Chem.*, 1911-12, x, 15.

SPERM AS AN INHIBITANT OF MATURATION AND FERTILIZATION.

By G. H. A. CLOWES AND ESTHER GREISHEIMER.

(From the Biochemical Research Laboratory, Eli Lilly and Company, Indianapolis.)

Immature starfish eggs are prevented from maturing by treatment with butyric acid, exposure to heat, etc., the identical procedures which, after maturation, induce artificial parthenogenesis.

Similarly exposure of the immature eggs to sperm, even in small amounts, inhibits or retards maturation rendering the eggs unfertilizable or difficultly fertilizable by sperm, at a subsequent stage at which control eggs not previously treated with sperm are readily fertilized.

The best contrasts may be obtained by giving the immature eggs a preliminary exposure to a weak butyric acid solution which retards but does not prevent maturation. This treatment followed immediately by exposure to sperm causes a marked interference with maturation and subsequent fertilization as compared with eggs which receive the butyric treatment only. Heat followed by sperm gives similar results.

Inhibiting effects of sperm on immature eggs may be counteracted by a brief treatment with 0.001 N NaOH, but not ammonia. The similarity of inhibiting effect obtained by butyric acid and sperm separately or in combination, and the reversal of these effects by NaOH, suggest that inhibiting effect of sperm is attributable to production of acid on or adjacent to egg surface.

COLLOID CHEMISTRY OF SOAPS AND PROTEINS.

By MARTIN H. FISCHER.

(From the Physiological Laboratory, University of Cincinnati, Cincinnati.)

On the assumption that the polymerized amino-acids which we call proteins react with bases of various sorts to form salts in the same fashion in which fatty acids unite with bases of various sorts to form soaps, the colloid chemistry of the latter was studied to obtain light regarding the colloid behavior of the former.

The hydration capacity of the soaps varies with the basic radical and, in general, in the order: NH_4 , K, Na, Li, Mg, Ca, Fe, Cu, Hg. It varies also with the type of fatty acid, that standing lowest in any homologous series being least hydratable. When different series are compared, the less saturated possess the lower hydration capacity.

Colloid soap jellies may be prepared from the various soaps and anhydrous solvents, like monatomic, diatomic, and triatomic alcohols, aldehydes, benzene, toluene, etc. This finding speaks against the too heavy emphasis now being placed upon purely electrical notions of stabilization in colloid systems.

Hydrophilic or lyophilic colloids are defined as dispersions, coarser than molecular, of one material in a second with the dispersed substance a solvent for the dispersion medium.

Liquid soaps may be "coagulated" by alkalies and neutral salts of various kinds even when chemical reaction between soap and added substances is impossible. The consecutive changes of setting, secondary liquefaction with progressive dehydration of the soap, and its separation in "coagulated" form are explained on the basis of union between added salt and water with production of an emulsion of salt water in soap succeeded by one of soap in salt water and ending in anhydrous soap floating upon salt water.

Chemically neutral soaps do not affect an indicator like phenolphthalein in concentrated solution. On diluting with water they turn it bright red. This means either (1) that water-in-colloid systems (analogous to normal cells and body fluids) cannot be properly analyzed by indicator methods but only colloid-in-water systems (analogous to the secretions from the body) or (2) that the former contain no ions.

From qualitative and quantitative analogy in colloid behavior between soaps and protoplasm the latter is held, in essence, to be a salt compound of various polymerized amino-acids with potassium, sodium, magnesium, calcium, etc., which materials have "dissolved" a certain amount of water. Introduction of acid, ammonia, or potassium into this compound represents poisoning by these materials at one end of such a soap-like series (as evidenced by increased hydration capacity and increased solubility in water); introduction of the heavy metals poisoning at

the other end (as evidenced by a decreased hydration capacity and the production of difficultly soluble compounds). Just as a soap anywhere in the series can, by appropriate means, be converted into any other, just so can a normal protein or living cell be "poisoned" through the addition of materials found at either extreme in the base series; or after such poisoning be changed to nearer the normal by addition of the bases in the middle series.

PATHOGENESIS DUE TO VITAMINE DEFICIENCY IN THE RAT.

BY A. D. EMMETT AND FLOYD P. ALLEN.

(From the Research Laboratory of Parke, Davis and Company, Detroit.)

The rats were fed on definite dietary planes which would produce normal growth except for the lack of either vitamine A or B. From these groups, animals were selected which were representative. Care was taken to see that the animals were free from infection. Control tests were made on tissues from rats fed a complete diet.

The various tissues examined histologically were: thymus, thyroid, pancreas, testes, ovaries, adrenals, liver, spleen, ileum, colon, kidneys, heart, lungs, brain, optics, lower cord, and sciatic nerves. In the absence of vitamine B, the most noticeable changes were found in the almost complete atrophy of the thymus; hypertrophy of the adrenals; passive congestion; fatty infiltration; and at times fatty degeneration in the liver and some atrophy of muscle fibers of the heart. There was more or less passive congestion in the pancreas, spleen, ileum, colon, kidneys, and lungs. In the rats which lacked vitamine A in their diet, there were no special outstanding pathological findings. In marked contrast with the lack of vitamine B the livers showed no fatty changes, the adrenals no hypertrophy, and the thymus no atrophy. The control animals proved to be normal as far as histological examination showed.

RELATION OF VITAMINES AND IODINE TO THE SIZE AND DEVELOPMENT OF THE TADPOLE.

By A. D. EMMETT, FLOYD P. ALLEN, AND MARGUERITE STURTEVANT.

(From the Research Laboratory of Parke, Davis and Company, Detroit.)

In the light of our previous work, the temperature conditions, the exact food control, and the question of individual variation were especially borne in mind by keeping the tadpoles in an environment where the temperatures could be maintained within reasonable limits, and by keeping each tadpole in a separate vessel so that there could be no possibility of their eating each other.

The results confirmed the previous findings in regard to the size of the tadpoles; namely, that the presence of vitamins bore a direct relation to size, the water-soluble type B being more of a factor than the fat-soluble A. With respect to the development of the hind legs, the tadpoles having the vitamins in their diet did better than those without them. Vitamine B was apparently more essential than vitamine A. However, for the completion of the metamorphosis, iodine in the inorganic form or in the organic form as in the desiccated thyroid gland was essential. The relative values referred to were determined from time to time by measuring the total body length from shadow photographs, and by binocular observation every 10 to 14 days of the stage of development of the hind legs. Calculating the arithmetic mean for each group on the date observed served as the guide to the rate of advancement.

PENTOSE CONTENT OF SOME TISSUES OF MARINE ANIMALS.

By C. BERKELEY.

(From the Marine Biological Station, Nanaimo, B. C.)

The prevalence of complex pentosans and methylpentosans in marine algæ make it a matter of interest to examine the tissues of animals whose diet they make up for compounds of the same class.

Many observations have been made on the occurrence of pentoses in animal tissues¹³ but the only systematic survey of the pentose content of various organs in one animal (the ox) is that by Grund.¹⁴ Although guanylic acid and inosinic acid are the only compounds in which it has been shown with certainty that the pentose exists certain workers have stated that complex pentosans or methylpentosans are present in certain mollusks living on algæ.

An examination of various tissues from a variety of marine animals indicated the presence of a substance soluble in boiling water, which while it yielded furfural was not precipitated by acetic acid from aqueous solution.

Before undertaking a further investigation of the nature of its combination it was thought important to ascertain the relative amounts of pentose in various marine animals.

For this purpose the tissues were first of all thoroughly extracted with alcohol and the air-dried residue was treated by Grund's modification of the method of Tollens and Kröbe for the determination of pentoses, the result being calculated as xylose, although it is probable that most if not all of the pentose is really present as *d*-ribose.

The following amounts of pentose were obtained: *Squalus* (dogfish) pancreas 2.28, spleen 0.67, kidney 0.65, liver 0.72, testis 0.92, heart 0.38, muscle 0.35 per cent; *Thais lamellosa* (gastropod) liver and gonads 1.15 per cent; *Evasterias troschelii* (starfish) liver 0.77 per cent.

Comparison of the results for the dogfish and for the ox as obtained by Grund show approximate correspondence, the most important difference being that there is relatively more pentose in the organs of the former except in the case of the pancreas, where the amounts are about the same in the two animals.

That the relatively high percentage in the liver (and gonads) of *Thais* may indicate that some is present in a polymerized form, is suggested by Röhmman and others for allied species.

¹³ For a summary of literature see Jones, W., *Nucleic acids: Their chemical properties and physiological conduct*, New York, 1914, 8, 33, 34.

¹⁴ Grund, G., *Z. physiol. Chem.*, 1902, xxxv, 111.

**PENTOSAN- AND METHYLPENTOSAN-SPLITTING ENZYMES OF
MACROCYSTIS PYRIFERA.**

By C. BERKELEY.

(From the Marine Biological Station, Nanaimo, B. C.)

By the spontaneous fermentation of the giant kelp (*Macrocystis pyrifera*) in the presence of ground limestone, the kelp almost disappears in 2 to 3 weeks and the solution contains considerable quantities of the calcium salts of mono-basic fatty acids.

No free sugar occurs in the plant. The only carbohydrate constituents present in sufficient quantity to account for the acids produced are pentosan and methylpentosan complexes, the former soluble in dilute alkali and precipitated on acidification, the latter soluble in water and precipitated by alcohol.¹⁵ Breakdown of these complexes to simple sugars must precede acid formation, but no such sugar can be detected when the fermentation takes its normal course.

If freshly cut kelp is extracted with boiling water the extract is neutral, has no reducing properties, and contains methylpentosan, but no pentosan. If the kelp has been cut into small pieces a few hours the extract is acid, reduces Fehling's solution, and contains pentosan as well as methylpentosan.

If kelp is kept for a day or so in strong alcohol and subsequently steeped in warm water containing toluene or other antiseptic, free sugar develops rapidly and both pentose and methylpentose are found in solution. After more prolonged contact with alcohol no breakdown of the polysaccharides occurs on subsequently putting the kelp into water.

By extracting fresh kelp rapidly with frequent changes of alcohol, drying at a low temperature, and grinding, a preparation can be obtained which produces free sugar very rapidly on being added to a solution of either the pentosan or methylpentosan complex. No formation of acid occurs if an antiseptic is present.

From these observations the following conclusions are drawn:

1. Kelp contains enzymes which start to break down its polysaccharide constituents as soon as it is cut.

¹⁵ Hoagland, D. R., and Lieb, L. L., *J. Biol. Chem.*, 1915, xxiii, 287.

2. The enzymes are inactivated by prolonged contact with alcohol.

3. The production of acids sets in, in the normal course of fermentation, as soon as the sugars are set free and is probably due to the action of bacteria adherent to the kelp.

DIRECT DETERMINATION OF SODIUM IN TISSUES AS SODIUM-CESIUM-BISMUTH-NITRITE.

By R. D. BELL AND E. A. DOISY.

(*From the Laboratories of Biological Chemistry, Washington University
Medical School, St. Louis.*)

The insolubility of sodium-cesium-bismuth-nitrite, previously described by Ball,¹⁶ was utilized in the development of a method for the determination of sodium in tissues.

The organic matter is oxidized by a wet ash process with a mixture of nitric and sulfuric acids. Iron must be removed from the mixture of blood salts. The solution is made faintly alkaline to methyl orange and evaporated to 2 or 3 cc. 0.5 cc. of 2 N HNO₃ and an excess of reagent are added. After standing 2 days under illuminating gas, the precipitate is filtered off on a previously weighed Gooch crucible. The reagent is removed by washing with acetone. The crucible is dried at 100° and weighed.

Since the insoluble salt is a nitrite, it may be estimated by titration with potassium permanganate. 1 molecule requires 30 atoms of oxygen for oxidation.

The precipitate contains 3.675 per cent of sodium. 1 mg. of sodium yields 27.2 mg. of the insoluble nitrite.

The reagent is made from cesium nitrate, bismuth nitrate, and potassium nitrite. Any insoluble matter which forms is dissolved by the addition of a little dilute nitric acid.

Good results were obtained in the determination of known amounts of sodium. There was good agreement between results by this method and the indirect perchlorate procedure on blood and urine samples.

¹⁶ Ball, W. C., *J. Chem. Soc.*, 1910, xcvi, 1408.

UROCHROME EXCRETION AS INFLUENCED BY DIET.

By K. F. PELKAN.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

Urochrome has long been known as a constituent of urine but its significance has never been definitely determined. Various workers have claimed its relation to the blood and bile colors and to the cellular metabolism of the organism and have denied that it was at all influenced by diet. The amount of experimental evidence submitted in favor of these claims has been small. The present work was undertaken to determine whether and to what extent the amount of urochrome excreted was dependent on the diet. Experiments carried out on a human subject resulted as follows: A low protein diet reduced the urochrome excretion up to 50 per cent, while a high protein diet raised it perceptibly. Gelatin added to a low protein diet did not affect the excretion. Colored substances were found in peptone and in amino-acid digestion mixtures which were similar in all respects to urochrome. All evidence points to the fact that urochrome is to a large extent derived from the proteins of the diet.

RELATION OF EXTRACT OF LUNG TO THE CLOTTING OF BLOOD.

By C. A. MILLS.

(From the Department of Biochemistry, University of Cincinnati, Cincinnati.)

Whereas lung extract, if injected rapidly and in sufficient amounts into animals, causes extensive intravascular coagulation and death usually in less than 1 minute, if injected in small amounts at first and then repeatedly with increasingly larger doses, causes the development of a negative coagulative phase in the blood of the animal. Such blood, withdrawn from the vessels, will not clot spontaneously for days at 5°C., putrefaction usually setting in before clotting occurs. Neither will it clot on treatment with more of the lung extract, calcium salts, thrombin, serum, CO₂ gas, or dilution with water. It differs from peptone plasma in its non-coagulability in that it contains no antithrombin or other material to inhibit the clotting of

normal blood or oxalate plasma when added to it, but instead it actually possesses a strong thromboplastic action, probably due to the presence of unchanged lung extract in it.

As much as 1,150 cc. of lung extract have been injected into an 11 kilo dog without causing intravascular clotting, whereas 3 or 4 cc. would kill a dog of this size if injected rapidly in a single dose.

The non-coagulability is not due to the disappearance of fibrinogen from the blood, for the addition of an equal volume of saturated NaCl solution to this plasma always produced a precipitate of fibrinogen. Probably the fibrinogen has been altered by the injections, since it no longer clots with thrombin.

THE RÔLE OF PHOSPHORIC ACID IN CARBOHYDRATE METABOLISM.

By CYRUS H. FISKE.

(From the Biochemical Laboratory, Harvard Medical School, Boston.)

The hourly inorganic phosphate output in man falls and later rises during the morning in the absence of food. After the administration of sucrose while the rate of phosphate excretion is decreasing, the fall is much more marked. The ingestion of sucrose while the rate of phosphate excretion is increasing is likewise followed by a drop in the phosphate output. In either case there is later a compensatory rise, indicating that carbohydrate combustion is accompanied by the retention of phosphate, which is later excreted at an exaggerated rate. It is suggested that this may be an indication that sugar is decomposed in the body *via* a phosphoric acid ester, as in alcoholic fermentation by yeast. The observation may account for the phosphate retention after parathyroidectomy, since the tetany caused by this operation is accompanied by rapid loss of stored carbohydrate.

UREA IN DOGFISH AND SKATE.

By C. C. BENSON.

(From the Biological Board of Control, and the Department of Food Chemistry, University of Toronto, Toronto.)

Determinations of urea in the muscle of dogfish and skate were made on fresh material, on material kept for varying times in cold storage and in the household refrigerator, and on material taken from cold storage and boiled.

In most cases, alcoholic solutions were used for analyses, using methods similar to those of Greene¹⁷ and in others the ammonia was aerated directly.¹⁸

Fresh dogfish gave 0.5 to 0.6 per cent of nitrogen from urea, specimens from cold storage, 0.4 to 0.57 per cent of urea nitrogen, and boiled specimens showed practically no loss of urea.

Specimens of the muscle of skate, fresh or directly after taking from cold storage, gave 0.6 to 0.8 per cent of urea nitrogen but this value decreased rapidly on keeping in the refrigerator.

The quantities of urea in both forms are too small to be injurious as food, both forms keep well in cold storage, but the skate rapidly forms ammonia from its urea on being thawed.

COMPOSITION OF THE TUBERS, SKINS, AND SPROUTS OF THREE VARIETIES OF POTATOES.

By F. C. COOK.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

Data collected for 20 years by the Vermont, Maine, and New York State Experiment Stations show that an increased yield of potatoes results from spraying potato vines with Bordeaux mixture. This is true whether any *Phytophthora infestans* (late blight of the potato) is prevalent or not.

Because of the high price of copper sulfate in 1915, experiments extending over four seasons were inaugurated by the Department of Agriculture comparing the fungicidal properties of

¹⁷ Greene, C. W., *J. Biol. Chem.*, 1919, xxxix, 435.

¹⁸ Sumner, J. B., *J. Biol. Chem.*, 1916, xxvii, 95.

Pickering sprays (made with saturated lime water and less copper sulfate than Bordeaux) with standard Bordeaux spray.

Results with Pickering and Bordeaux sprays on two varieties of tubers at Arlington, Virginia, the past season showed an apparent increased yield of tubers and an increased percentage of solids compared with the control. Bordeaux-sprayed potatoes from several states the past season also showed increased yields and increased percentage of solids of the tubers. Green Mountain, Rural New Yorker, and Irish Cobbler tubers stored in the laboratory (average temperature 70°C.) from the time of digging in September, 1918, until sprouting had proceeded to the limit in the Spring of 1919, showed that the sprouts comprised respectively 7.2, 3.5, and 13.3 per cent of the total weight of the tubers, skins, and sprouts. A difference in the concentration or activity of the growth-promoting agencies is suggested. The analytical data for the sprouts, skins, and tubers of these and other samples show little variation in composition for the different varieties of potatoes.

Analyses of sprayed and unsprayed Green Mountain tubers, skins, and sprouts indicate that the spray did not alter the rate of growth or the composition of the sprouts.

The percentage distribution of the nitrogenous substances showed that the sprouts contained more protein, less water-soluble, about the same monoamino and amide nitrogen, and less diamino and other basic nitrogen than the tubers. The sprouts showed a selective action in withdrawing nitrogen, total ash, phosphorus, and water from the tubers in larger proportion than these constituents existed in the tubers.

GLYCOSURIA IN THREE CASES OF CHRONIC NEPHRITIS WITH EDEMA BUT WITH ONLY SLIGHT NITROGEN RETENTION.

BY VICTOR C. MYERS AND LUDWIG KAST.

(From the Laboratory of Pathological Chemistry and the Department of Medicine, New York Post-Graduate Medical School and Hospital, New York.)

So called "renal diabetes" has been the subject of considerable discussion since Lépine in 1895 postulated the existence of this rather interesting condition, in which the glycosuria is actually

the result of renal disease, and not due to hyperglycemia as in diabetes mellitus. About thirty cases have now been recorded in the literature. A few of these cases have shown definite evidence of renal disease aside from the glycosuria, although some would appear to be entirely free from the symptoms ordinarily associated with disease of the kidney. Renal diabetes has been compared with phlorhizin glycosuria, in which condition we have glycosuria without hyperglycemia. Some of the cases, and especially those which we wish to report, find a more direct analogy in the glycosuria of uranium nephritis. Here there is only a mild glycosuria, with a normal or nearly normal glycemia, and a constant proteinuria. Ryffel¹⁹ and Roger²⁰ have each reported a case similar in some respects to our own. Our three cases (two males, aged 54 and 62, and one female, aged 10) all showed marked proteinuria and edema, but little nitrogen retention, urea nitrogen figures of 11 to 29 mg. per 100 cc. of blood. One of the cases gave a normal blood sugar, but the other two showed a mild hyperglycemia such as is ordinarily encountered in severe nephritis. In one of these the glycosuria was shown by Bailey²¹ to be entirely independent of the slight hyperglycemia. The figures for the urine sugar content ranged from negative findings to slightly over 2 per cent, although when sugar was present, it most often amounted to about 0.5 per cent.

**A SIMPLIFIED METHOD FOR THE ESTIMATION OF MORPHINE
TOGETHER WITH DATA ON THE DISTRIBUTION OF THE
ALKALOID IN ACUTE POISONING.**

By SERGIUS MORGULIS AND VICTOR E. LEVINE.

(From the Biochemical Laboratory, College of Medicine, Creighton
University, Omaha.)

Morphine can conveniently be determined in food, tissue, or body fluid by heating with 2 per cent tartaric acid (if solid, the material should first be ground or finely minced) to convert all morphine into the soluble tartarate. The mixture is rapidly cooled, preferably on ice, to solidify the fatty material. The

¹⁹ Ryffel, J. H., *Quart. J. Med.*, 1915-16, ix, 91.

²⁰ Roger, H., *Presse méd.*, 1917, xxv, 337.

²¹ Bailey, C. V., *Am. J. Med. Sc.*, 1919, clvii, 221.

solid residue is removed by straining through cheese-cloth, and washed until the washings are no longer acid to litmus. The liquid, after being filtered through paper, is evaporated to a pasty consistency. The tartarate is then decomposed by the addition of an excess of solid sodium bicarbonate, which sets the alkaloid free. The evaporation is then continued to complete dryness, and the mass powdered and extracted with chloroform to remove the free morphine. The volume of the chloroform extract is noted, and the smallest quantity of the extract is found which on evaporation (in a porcelain crucible over water bath) leaves a residue which yields a definite morphine test. In this way, the relative amount of morphine in several extracts can be determined; knowing the limit of sensitivity of the reaction an approximate estimate of the amount of morphine in the original sample is possible.

The various alkaloidal tests can be applied to the residues after the evaporation of portions of the chloroform extract. The reagent employed—selenium dioxide dissolved in concentrated sulfuric acid—is very sensitive towards the opium alkaloids. It is more sensitive than many of the older reagents.

From experiments on rabbits with acute poisoning the conclusion drawn is that morphine, whether given subcutaneously or by mouth, is widely distributed throughout the animal body, finding its way into almost every tissue. The morphine is invariably found in appreciable quantities in the urine and kidney. Also large quantities may be present in the alimentary tract, liver, lungs, and brain. According to our results especially large amounts of morphine were present in the alimentary tract and excretory organs after administering the poison by mouth, while after injecting under the skin it was recovered principally from the liver, excretory organs, and also from lungs and brain. In view of these findings, it is not feasible to limit the toxicological examination for morphine to the alimentary tract *alone*. An examination of at least the kidney, urine, and liver is also indispensable.

**ON THE PROTECTION AGAINST EOSIN HEMOLYSIS AFFORDED
BY CERTAIN SUBSTANCES.**

By C. L. A. SCHMIDT AND G. F. NORMAN.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

It was found that the inability of gelatin to afford any protection against the photodynamic action of eosin on red cells while marked protection is shown by blood serum, casein, edestin, and certain other proteins can be directly attributed to the lack of tyrosine and tryptophane, since the amino-acids can themselves afford marked protection. The presence of the benzene ring in a molecule does not confer protective ability while the hydroxyphenyl group appears to be one of the determining factors.

EFFECT OF ANESTHETICS ON CELL RESPIRATION.

By J. F. McCLENDON.

(From the Physiological Laboratory, University of Minnesota Medical School, Minneapolis.)

Owing to the difficulties encountered in keeping the metabolism down to the basal level in higher animals, comparative studies in metabolism are desirable. In choosing an animal for such investigations the jellyfish *Cassiopea xamachana* was decided on since the automatic activity of the nervous system may be abolished by cutting off the margin of the bell. The respiration rate is independent of the oxygen tension except at extremely low tensions of oxygen. In determining the rate of respiration, four jellyfish of large size (up to 15 cm. diameter) were deprived of manubrium and bell-margin and placed in a water-tight jar of about a liter capacity and rotated in a thermostat (kept at 30°) for 1 hour. The jar was filled up with sea water of known O₂ and CO₂ content, with the exclusion of air bubbles. The O₂ was determined by the Winkler method and the CO₂ calculated from the alkaline reserve and pH. It was found that the nervous conduction in the bell was anesthetized by 0.5 per cent ether. The jellyfish died at the end of 1 hour in 3 per cent ether, and in less than 1 hour in 4 per cent ether. The respiratory quotient

was found to be about 0.95, and since the oxygen could be determined more accurately than the CO_2 , only the former will be given. The respiration was measured for 1 hour before anesthetization as a control.

	Oxygen (O_2).							
	0.5 per cent.	1 per cent.				2 per cent.		3 per cent.
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Ether	2.60	2.40	1.80	2.70	1.80	2.20	1.20	1.20
Control	2.65	2.40	1.80	2.65	2.20	2.10	1.20	2.05

Within the limit of error of the apparatus the O_2 consumption was the same with or without ether, up to 4 per cent in which the jellyfish died before the end of the hour. Carbon dioxide is sometimes considered an anesthetic for marine animals. The following experiments were made by adding CO_2 to sea water of 0.0025 N alkaline reserve and determining the pH at the beginning and end of the experiment and recording the mean value. The pH for the control was always 8.15.

	Oxygen (O_2).				
	pH 6.60.	pH 6.30.	pH 5.80.	pH 5.70.	pH 5.50.
	cc.	cc.	cc.	cc.	cc.
After CO_2	2.00	1.85	1.90	0.70	0.40
Control	1.08	2.05	2.60	1.70	1.70

The addition of CO_2 reduced respiration, but the question arises whether the undissociated CO_2 molecules or the H ions were responsible for this effect. In the following experiments no CO_2 was added to the sea water, but HCl was added thus changing the character of the ionization of the "total CO_2 " already present, as indicated by the change in pH. The pH of the control was 8.15.

	Oxygen (O_2).	
	pH 6.60.	pH 5.80.
	cc.	cc.
After HCl	2.02	1.30
Control	2.07	1.85

These experiments show that the CO_2 liberated from the carbonate and bicarbonate by the addition of HCl lowers the metabolism slightly. In order to show the possible effect of change in pH without increasing undissociated CO_2 , acid was added to sea water and the CO_2 expelled. The pH was 5.8, and 2.9 cc. of O_2 were used as compared with a control of 2.7 cc. of O_2 in normal sea water of $\text{pH} = 8.15$. Since this is within the limit of error of the apparatus we presume that the H ions were not responsible for the inhibitory effect of CO_2 on respiration of the cells covering the surface of the bell of the jellyfish.

A METHOD OF INCREASING THE SHARPNESS OF THE TONE-MINIMUM IN MEASURING THE ELECTRIC CONDUCTIVITY OF CELLS.

By J. F. McCLENDON.

(From the Physiological Laboratory, University of Minnesota Medical School, Minneapolis.)

In measuring the electric conductivity of salt solutions, the sharpness of the tone-minimum is increased by shunting a condenser across the resistance box. Variable condensers sold for that purpose have a maximum capacity of about 0.003 microfarads. Our conductivity cell, when filled with distilled water, has a capacity of less than 0.003 microfarads and a condenser of that capacity may be used with it. When filled with blood, however, such a condenser is entirely inadequate, and owing to the great cost of large variable condensers a home-made apparatus was constructed to increase the sharpness of the tone-minimum. Since this title was sent in, however, we have found that a variable condenser may be built up of telephone condensers at a moderate cost. When our cell was filled with blood a capacity of 1.3 microfarads was required across the box, whereas on the addition of saponin to lake the blood a capacity of 2.2 microfarads was required.

**RELATION BETWEEN CHOLESTEROL AND CHOLESTEROL
ESTERS IN THE BLOOD DURING THEIR ABSORPTION.**

BY ARTHUR KNUDSON.

*(From the Laboratory of Biological Chemistry, Union University, Medical
Department, Albany Medical College, Albany.)*

A series of experiments were carried out on dogs, feeding them cholesterol and cholesterol esters, respectively, to determine the relation between the two in the blood during their absorption. In a previous paper, it was shown that, during absorption of a neutral fat (olive oil), there was a marked increase of the cholesterol esters in both the plasma and the corpuscles but no significant change in the total cholesterol.

Cholesterol was determined by the Bloor method, and cholesterol esters by the Bloor and Knudson method. In all the experiments, fat was excluded from the diet. Five experiments were studied in which from 3 to 4 gm. of cholesterol were fed to dogs, and blood specimens taken every 2 hours up to 8 hours after feeding. There was found to be a marked rise in the total cholesterol both in the plasma and in the corpuscles, and in three of the five experiments the increase in the corpuscles was greater. The amount of cholesterol in the combined form as esters remained constant throughout these experiments, indicating that the cholesterol was absorbed in the free form. In a series of four experiments feeding cholesterol esters (such as palmitate, stearate, and oleate) similar increases of the total cholesterol of the plasma and corpuscles were obtained. In three of the four experiments, the increase in total cholesterol was greater in the corpuscles. There was no change in the cholesterol esters in these experiments, indicating that the cholesterol esters must have been hydrolyzed probably in the intestines before being absorbed and that they did not combine again before entering the blood.

**THE FORMATION OF ACETONE BODIES FOLLOWING ETHER
ANESTHESIA AND THEIR RELATION TO THE
PLASMA BICARBONATE.**

BY JAMES J. SHORT.

*(From the Laboratory of Pathological Chemistry, New York Post-Graduate
Medical School and Hospital, New York.)*

A study of the acetone bodies of the blood and urine was made in twelve cases with the gravimetric methods of Van Slyke and Fitz for blood and Van Slyke for urine. The blood content was but little affected during an average period of 48 minutes of ether anesthesia, but in two cases examined at intervals over a longer period there was a slight increase a few hours later and an increased output in the urine. The CO_2 -combining powers in these cases increased even during increase of β -hydroxybutyric acid.

Experimental data indicate that fat in the presence of ether and ether itself may interfere with the accuracy of the method, since both may form a precipitate with the reagents and thus increase the weight of the precipitate formed from the acetone bodies. It could not be shown from analyses on postoperative blood specimens that ether directly effected much error, but through its ability to penetrate the filter it apparently carried with it some fat in solution which caused the increase. Glycerol formed a comparatively large precipitate with the reagents, oleic acid a much smaller precipitate.

It was concluded that, in the cases reported, acetone bodies were not formed promptly enough during ether anesthesia to account for the decreased plasma bicarbonate and that results of analyses for β -hydroxybutyric acid on postoperative bloods may in some instances have been too high, due to an error introduced as a result of the ether content.

PROPERTIES OF HEMOCYANIN AS AN ANTIGEN.

By C. L. A. SCHMIDT.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

Hemocyanin obtained from the abalone was found to be precipitated by ammonium sulfate within the limits given by Alsberg for that obtained from *Limulus*. On repeated injections into rabbits, positive fixation and precipitin tests were obtained. The substance in moderate doses is non-toxic for guinea pigs.

ENERGY ELIMINATION AND GASEOUS EXCHANGE IN BANANAS, PINEAPPLES, AND APPLES.

By C. F. LANGWORTHY, R. D. MILNER, AND H. G. BAROTT.

(From the Office of Home Economics, States Relation Service, United States Department of Agriculture, Washington.)

The changes in ripening fruit are due to enzymes normally present in the fruit and vary with the condition of the surrounding atmosphere in respect to heat, light, water vapor, and other gases. The practical improvement of storage conditions can best be secured by exact knowledge of the biological changes in the materials stored, which in turn implies an increased knowledge of the atmospheric factors just mentioned. The respiration calorimeter in this laboratory is well adapted for the study of such problems and has been used in experiments with apples, bananas, and pineapples. The bananas and pineapples were in the state known in the trade as "active ripening" while the apples were a Fall variety, recently harvested for storage for winter use.

The following table summarizes the results.

Hourly Energy Elimination and Gaseous Exchange per Kilo of Fruit Stored at Room Temperature (10-30°C.).

Fruit.	Energy.	Oxygen.	Carbon dioxide.	Water vapor.	Specific heat.
	<i>cal.</i>	<i>liters</i>	<i>gm.</i>	<i>gm.</i>	
Bananas	0.31	0.06	0.12	0.35	
Pineapples.....	0.19	0.03	0.07	0.14	
Apples	0.04				0.93

That the changes thus measured are of considerable magnitude appears from a comparison with the energy elimination of the human body at complete rest, which is estimated as 1.1 calories per kilo per hour.

INDICAN IN THE SALIVA OF A CASE OF PELLAGRA COMPLICATED WITH TUBERCULOSIS.

By M. X. SULLIVAN.

(From the Pellagra Hospital, United States Public Health Service, Spartanburg.)

The urine of many of the patients at the hospital was found to contain a high content of indican. Tests were made on the saliva to see if indican could be found therein, using the Obermayer reagent and extracting with chloroform. In Case 313 with large amounts of indican in the urine indican was found likewise in the saliva in two tests. Once the chloroform was blue with indigo blue and once red with indigo red. No other saliva showed the presence of appreciable amounts of indican. It may be noted that shortly after the discovery of indican in his saliva, Case 313 was judged to be suffering from tuberculosis also, from which disease he died about a month later. What part the tuberculosis played in the presence of indican in the saliva cannot be told.

BIOCHEMICAL STUDIES OF THE SALIVA IN PELLAGRA.

By M. X. SULLIVAN AND K. K. JONES.

(From the Pellagra Hospital, United States Public Health Service, Spartanburg.)

In pellagra the condition of the mouth, and especially of the tongue, is of considerable importance in establishing a correct diagnosis. The true pellagrous tongue is vividly red and more or less swollen. The literature also speaks of salivation as a symptom of pellagra. In careful quantitative studies at the Pellagra Hospital, Spartanburg, South Carolina, some interesting data were gathered on the saliva in pellagra. It was found that, though there were cases of increased salivary flow, the salivation spoken of by the patients was often apparent rather than real

and was seemingly due to some inhibition of swallowing combined with a peculiar ropy change in the saliva or a high content of mucus which made the presence of saliva in the mouth more obvious. Occasionally also the flow was very slow, but in general it was within normal limits which vary considerably. The specific gravity of the saliva of the pellagra patients tended to be higher than that of the controls. The total solids, ash, organic matter, and mucin of the saliva was greater for the pellagrins than for the controls, but bore no relation to the mouth symptoms. The diastatic power of the saliva of pellagrins varied within the limits established by the controls. The sulfocyanate content was much less marked in the saliva of the pellagra patients than in that of normal people. The reaction of the saliva in pellagra was found to be somewhat more alkaline than that of normal saliva.

IS FIBRINOGEN FORMED IN THE LIVER?

By A. P. MATHEWS.

THE ANTIKETOGENIC ACTION OF GLUCOSE.

By P. A. SHAFFER.

A NEW QUALITATIVE AND QUANTITATIVE COLOR REACTION FOR AMINO-ACIDS.

By OTTO FOLIN AND H. WU.

ON THE DETERMINATION OF BLOOD SUGAR.

By S. R. BENEDICT AND ELIZABETH FRANKE.

A CONVENIENT PERMANENT UREASE PREPARATION.

By OTTO FOLIN.

ON THE LIPINS OF HUMAN CORPUS LUTEUM.

By JACOB ROSENBLOOM.

OBSERVATIONS ON THE CHEMICAL PATHOLOGY OF THE BLOOD IN PERNICIOUS ANEMIA.

By AMOS W. PETERS AND A. S. RUBNITZ.

THE EFFECTS OF MODERATE HEMORRHAGE, ETC., ON SOME OF THE BLOOD ELEMENTS AND CONSTITUENTS.

By E. S. SUNDSTROEM AND W. R. BLOOR.



THE DIGESTIBILITY OF CHICKEN SKIN.

By EDWARD F. KOHMAN AND H. A. SHONLE.

(From the Food Research Laboratory, Bureau of Chemistry, Department of Agriculture, Indianapolis.)

(Received for publication, February 3, 1920.)

While certain feeding experiments were being conducted in poultry fattening by the use of various feed by-products, an opportunity presented itself to collect a considerable quantity of chicken skin. Advantage was taken of this to carry out two metabolism experiments to determine the digestibility of this food product. From 10 to 15 per cent of the edible nitrogen in the chicken is found in the form of skin. The fat content of this skin may vary from a very small percentage to as high as 50 per cent or even more, counting the loose layers of fat lying next to the muscular tissue as part of the skin, as was done in the experiments alluded to above.

In order to remove part of this fat to make the skin more suitable to the metabolism experiments, the ground skin was heated in a water bath and then the larger part of the fat was expressed through a cloth bag. In this manner considerable water extract was also expressed, which gelatinized under the layer of fat upon cooling. This extract was combined with the skin from which it had been expressed and the whole thoroughly mixed again. The skin as then used for the experiments had 26.3 per cent fat and 3.03 per cent nitrogen. To prepare it for eating it was rolled in balls and fried. In this form it was a fairly palatable dish. It can be calculated from Tables I and II that during the experimental period this skin furnished 67.5 per cent of the nitrogen in the diet for Subject A and 65.1 per cent for Subject B.

It should be said of the chicken skin used in the experiments that it came from broilers and roasters as they came from the

range to the feeding station and from similar birds after a 2 weeks period of fattening on a buttermilk mash. The fattened birds were in the majority, and, as they were all commercially dry picked, the skin had an abundance of pin feathers.

The details of the experiments are given in Tables I to V. Each period consisted of 5 days, the experimental period being preceded and followed by a like period with the exception that milk, eggs, and meat took the place of the chicken skin. Other-

TABLE I.
Food Intake, Subject A, Man, Weight 180 Lbs.

Food.	Fore period.		Experimental period.		After period.	
	Food.	Nitrogen.	Food.	Nitrogen.	Food.	Nitrogen.
	gm.	gm.	gm.	gm.	gm.	gm.
Apples.....	1,050	0.26	1,050	0.26	1,050	0.26
Prunes.....	450	0.50	450	0.50	450	0.50
Oranges.....	1,189	1.53	1,189	1.53	1,213	1.57
Lettuce.....	240	0.46	200	0.38	200	0.38
Tomatoes.....	500	0.96	500	0.96	500	0.96
Sugar.....	514		324		108	
Tapioca.....	73	0.02	73	0.02	73	0.01
Bread.....	616	8.38	550	7.92	550	7.34
Potatoes.....	1,877	6.61	1,877	6.61	1,877	6.61
Butter.....	412	0.66	135	0.22	194	0.31
Eggs.....	496	10.61				
Meat.....	575	16.71			1,156	38.18
Milk.....	2,486	11.61				
Skin.....			1,260	38.18		
Total N.....		58.31		56.58		56.12
Total calories.....	14,697		11,589		8,792	

wise the diet was kept uniform throughout and an attempt was made to have it approximately normal for the individuals concerned. No attempt was made to limit the intake. As a result the caloric intake for the fore period was considerably higher than the after period due in part to the novelty of the experiment and the cold weather in the beginning, in part to the high fat content of the first lot of Hamburger steak purchased, and a combination of other circumstances. The feces for the different periods

TABLE II.
Food Intake, Subject B, Man, Weight 140 Lbs.

Food.	Fore period.		Experimental period.		After period.	
	Food.	Nitrogen.	Food.	Nitrogen.	Food.	Nitrogen.
	gm.	gm.	gm.	gm.	gm.	gm.
Apples.....	1,078	0.27	1,078	0.27	1,078	0.27
Prunes.....	562	0.62	562	0.62	450	0.50
Oranges.....	1,240	1.60	1,209	1.56	1,150	1.48
Lettuce.....	240	0.46	200	0.38	200	0.38
Tomatoes.....	500	0.96	500	0.96	500	0.96
Sugar.....	459		270		278	
Tapioca.....	85	0.02	85	0.02	85	0.01
Bread.....	776	10.55	686	9.88	722	9.60
Potatoes.....	2,403	8.48	2,403	8.48	2,403	8.48
Butter.....	482	0.77	371	0.59	242	0.39
Eggs.....	484	10.36				
Meat.....	670	19.45			1,285	42.42
Milk.....	3,820	17.23				
Skin.....			1,400	42.42		
Total N.....		70.77		65.18		64.49
Total calories.....	17,237		14,753		11,070	

TABLE III.
Utilization of Nitrogen.

	Subject A.			Subject B.		
	Fore period.	Experimental period.	After period.	Fore period.	Experimental period.	After period.
Urine, cc.....	6,985	8,125	9,210	7,395	6,970	8,185
Feces, gm.....	524	464	573	1,145	921	661
Urinary nitrogen, gm.....	38.33	47.69	54.04	55.30	59.90	61.99
Fecal " ".....	7.57	8.14	8.45	13.01	11.08	8.73
Nitrogen balance, ".....	+12.41	+0.75	-6.37	+2.46	-5.80	-6.11
Utilization, per cent.....	87.02	85.61	84.94	83.69	83.00	86.49

TABLE IV.
Fat Ingested.

Food.	Subject A.			Subject B.		
	Fore period.	Experi-mental period.	After period.	Fore period.	Experi-mental period.	After period.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Butter.....	350	115	165	410	316	206
Eggs.....	52			51		
Meat.....	109		74	128		82
Skin.....		332			369	
Milk.....	99			153		
Bread.....	8	7	7	10	9	9
Total.....	618	454	246	752	694	297

TABLE V.
Utilization of Fat.

	Subject A.			Subject B.		
	Fore period.	Experi-mental period.	After period.	Fore period.	Experi-mental period.	After period.
Dry matter in feces, <i>per cent</i>	25.91	25.64	23.28	17.85	14.96	20.86
Fat in dry matter, " "	12.80	12.17	9.84	13.44	15.06	7.84
" excreted, <i>gm.</i>	17.37	14.48	13.13	27.46	20.75	10.83
" utilized, <i>per cent.</i>	97.2	96.8	94.7	96.3	97.0	96.4

were marked off by means of lamp black. The vegetables and fruits were not analyzed, the composition given by Atwater and Bryant being used.¹

CONCLUSION.

In experiments in which 65.1 to 67.5 per cent of the nitrogen of the diet was supplied by chicken skin there was as good utilization of the nitrogen as when the same proportion of the nitrogen was supplied by meat, eggs, and milk.

¹Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Off. Exp. Stations, Bull. 28*, revised, 1906.

EFFECT OF SLEEP UPON THE ALKALI RESERVE OF THE PLASMA.

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(Received for publication, February 17, 1920.)

It has been shown by Leathes (1) that the CO_2 tension of alveolar air is definitely increased as a result of sleep. He is of the opinion that the respiratory center is depressed during sleep and that the morning alkaline tide in the urine, described by him, is due to increased respiratory activity on waking. That the respiratory center is depressed during sleep would seem quite possible. It is also to be expected, however, that with depression of the respiratory center there would occur a slight increase in the C_H of the blood. As the reaction of the blood is determined by the molecular ratio $\frac{\text{H}_2\text{CO}_3}{\text{NaHCO}_3}$ (2) it would follow, if the CO_2 tension of the alveolar air is increased without a proportionate rise in the blood bicarbonate, that the C_H of the blood would be slightly increased. In order to determine whether the plasma bicarbonate is altered during sleep, blood samples were taken for analysis by the Van Slyke method from nine students who kindly volunteered their services. The samples were taken in the evening shortly before the subjects retired and then again in the early morning, the subjects first being roused from a sound slumber. The blood was aspirated from an arm vein into a glass syringe containing a trace of potassium oxalate. It was transferred at once to a paraffined glass tube and centrifuged. The plasma was then saturated in a separating funnel with CO_2 at the tension of the alveolar air of the normal subject and the determination of the CO_2 content made by the method of Van Slyke and Cullen (3). The CO_2 bound as bicarbonate by 100 cc. of plasma was calculated and the results are shown in Table I.

TABLE I.

	CO ₂ bound as bicarbonate by 100 cc. of plasma.	
	Evening. 9.30 to 11 p.m.	Morning. 6 to 7.30 a.m.
	cc.	cc.
W. W. B.....	61	62
W. F. C.....	68	64
E. M. C.....	65	61
G. V. F.....	65	62
R. E. F.....	61	58
D. D. H.....	65	65
E. G. K.....	68	63
E. D. T.....	66	63
G. F. Y.....	67	67

DISCUSSION.

It was found in three cases that the bicarbonate level of the venous plasma was unaltered as a result of sleep, while in the remaining six instances there was a definite increase in this factor.

These results taken in conjunction with the observations of Leathes (1) that there is a definite rise in the alveolar CO₂ during sleep would indicate that the C_H of the blood is actually increased at this time. This increase in blood C_H may be effected in one of two ways, an increase in alveolar CO₂ without a relative increase in blood bicarbonate, or an increase in alveolar CO₂ with an actual fall in blood bicarbonate. One or the other of these types of regulation of blood C_H is manifested by the sleeping individual.

SUMMARY.

The alkali reserve of the blood plasma is either unaltered or depressed during sleep. An actual increase in the C_H of the blood during sleep is indicated.

I wish in conclusion to thank the students who offered their services, and Dr. P. L. Backus who assisted in making the analyses.

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2. Henderson, L. J., *Ergebn. Physiol.*, 1909, viii, 254.
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THE UTILIZATION OF α -METHYLGLUCOSIDE BY ASPERGILLUS NIGER.

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(Received for publication, January 27, 1920.)

The striking difference in the rate of utilization of α - and β -methylglucoside by *Aspergillus niger* was pointed out some years since by Dox and Neidig.¹

When a culture medium containing α -methylglucoside as the only source of carbon was inoculated with spores of *Aspergillus niger*, only a very meager growth of the fungus resulted. Even after several weeks the surface of the medium was only incompletely covered with mycelium in isolated colonies. On the other hand, the isomeric β -methylglucoside gave a dense mycelium which covered the entire surface. Examination of the substrate in both cases showed that in 6 days 100 per cent of the β -glucoside had disappeared, but only 1.2 per cent of the α -glucoside. Even after 20 days the latter had diminished by only 8.2 per cent. Experiments with enzyme preparations showed a similar difference in behavior toward the two isomers. Under practically the same conditions, 5.2 per cent of the α - and 85.5 per cent of the β -glucoside were hydrolyzed.

From these experiments it is apparent that α -methylglucoside can be utilized to a slight extent by this organism and that the enzyme necessary for its hydrolysis is produced in small amount under ordinary conditions. Attempts to bring about an adaptation of the fungus by transferring the mycelium to the glucoside solution after previous growth on sucrose did not result in any marked increase in availability of the substrate. The conditions under which this experiment was conducted were not such as to induce the maximum utilization of the substrate.

¹ Dox, A. W., and Neidig, R. E., *Biochem. Z.*, 1912, xlvii, 397.

This method of bringing about an apparent adaptation of an organism to a new substrate, by allowing the organism to attain a vigorous growth then replacing the original medium by the new substrate, was first employed by Pottevin.² In this way, Pottevin claims to have succeeded in inducing *Aspergillus niger* to secrete in demonstrable quantities the enzymes necessary for the hydrolysis of the two isomeric methylgalactosides. In his experiments, however, the substrate contained all the elements of the original culture medium. His experiments with methylgalactoside and our previous experiments with α -methylglucoside were therefore not strictly comparable. Subsequent experiments on autolysis of this organism, conducted by one of us,³ showed that replacement of the exhausted medium by a quantity of fresh medium resulted in a much denser growth and less autolysis than replacement of the medium by a solution of pure sucrose. It seems probable that the utilization of the new substrate depends to a considerable extent upon the presence of an available source of nitrogen, and perhaps also mineral salts.

EXPERIMENTAL.

The object of this work was to determine more precisely the extent to which *Aspergillus niger* can utilize α -methylglucoside under optimum conditions. As already stated, this substance when introduced into a culture medium as the only source of carbon, enables spores of *Aspergillus niger* to germinate and develop to a thin scanty mycelium which seems to direct most of its vitality toward the formation of spores. α -Methylglucoside is, however, not toxic since its presence in a medium containing sufficient sugar has no inhibitory effect upon growth. The following experiments demonstrate this fact quite clearly.

A culture medium was prepared according to Czapek,⁴ omitting the sucrose. 50 cc. of this were placed in each of three 200 cc. Jena flasks. To the first was added 2 per cent of sucrose, to the second 2 per cent of α -methylglucoside, and to the third 1 per cent of each of the preceding. The flasks were sterilized, inocu-

² Pottevin, H., *Ann. Inst. Pasteur*, 1903, xvii, 31.

³ Dox, A. W., *J. Biol. Chem.*, 1913-14, xvi, 479.

⁴ Czapek, F., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 47.

lated with spores of *Aspergillus niger*, and placed in an electric incubator at 35° until the resulting mycelium had become covered with spores. The mycelium was then filtered on Gooch crucibles, washed, dried, and weighed.

The filtrates gave no test for reducing sugar. It is apparent from Table I that in both Nos. 2 and 3 some of the glucoside has been utilized by the fungus. Medium 3, which contained only half as much sucrose as No. 1 and half as much glucoside as No. 2, yielded more mycelium than half the sum of those from Nos. 1 and 2.

TABLE I.

Medium No.	Weight of dry fungus.
	gm.
1	0.2779
2	0.0729
3	0.1830

The above experiment was repeated, using Raulin's medium upon which this organism thrives still better. No. 1 contained no glucoside, No. 2 contained 2 per cent of glucoside in place of the sucrose, and No. 3 contained 1 per cent of glucoside in addition to the full amount of sucrose.

TABLE II.

Medium No.	Weight of dry fungus.		
	1	2	Average.
	gm.	gm.	gm.
1	0.5906	0.5720	0.5813
2	0.0715	0.0851	0.0783
3	0.6970	0.7203	0.7087

The amount of glucoside utilized is evidently greater in No. 3 than in No. 2 (Table II). The probable explanation for this is the fact that after the fungus has attained a vigorous growth on the sucrose there is a much greater amount of mycelium to attack the glucoside than in the case of the scanty growth without the sucrose. Further experiments, recorded in Table III, apparently failed to confirm this observation, because the utilization of gluco-

side was carried practically to completion in all cases. The increase due to 1 per cent of glucoside is just about half the yield from 2 per cent of glucoside alone.

The effect of cultivating the fungus on Raulin's medium until vigorous growth and then replacing the medium by a similar medium in which α -methylglucoside is substituted, is quite striking. In the experiments recorded in Table IV the fungus

TABLE III.

Medium No.	Weight of dry fungus.					
	1	2	3	4	5	Average.
	gm.	gm.	gm.	gm.	gm.	gm.
1	0.9115	0.8924	0.8112	0.8574	0.8552	0.8655
2	0.1606	0.1423	0.1474	0.1421	0.1417	0.1468
3	1.0206	0.9755	0.9014	0.9045	0.8955	0.9393

TABLE IV.

Age of culture after transfer.	Before spore formation.		After spore formation.	
	Rotation of medium.	Decrease.	Rotation of medium.	Decrease.
days	°V.*	per cent	°V.	per cent
0	17.5	0.0	18.2	0.0
1	14.2	18.8	13.6	25.1
2	13.6	22.2	12.2	32.9
3	12.2	30.2	11.4	37.5
4	11.8	32.5	10.8	40.6
5	11.6	33.7	10.0	45.0
6	11.2	36.0	8.8	51.6
7	11.0	37.1	6.8	61.5
8	11.0	37.1	7.0	62.6

*°V. = degrees on Ventzke scale. For conversion to angular degrees multiply by 0.3468.

was first grown on Raulin's medium. In the first series the transfer was made just before and in the second series just after the formation of spores. The transfer of media was made by first syphoning off the medium and replacing twice with equal amounts of sterile distilled water, each time allowing the mycelium and water to remain in contact about 45 minutes, taking care not to wet the surface of the culture, and finally replacing the water

in medium containing glucoside as the sole source of carbon. On successive days the medium was removed from one of these flasks and an aliquot examined polarimetrically after clarification with neutral lead acetate. From the decrease in optical rotation the per cent loss of glucoside is calculated. The glucose was used by the fungus as fast as it was liberated from the glucoside, as is shown by the fact that the test with Fehling's solution was invariably negative. Qualitative tests for methyl alcohol were, however, positive.

As may be seen from Table IV, the more mature cultures utilize a larger percentage of glucoside in a given time. This

TABLE V.

Age of culture after transfer.	Before spore formation.		After spore formation.	
	Rotation of medium.	Decrease.	Rotation of medium.	Decrease.
days	°V.	per cent	°V.	per cent
0	17.9	00.0	17.7	00.0
1	13.4	25.1	14.0	20.9
2	11.2	37.4	12.4	29.9
3	9.0	49.7	11.6	34.4
4	9.0	49.7	10.6	40.1
5	7.0	60.9	7.8	56.4
6	5.8	67.6	5.2	70.6
7	4.4	75.4	4.8	72.1
8	2.8	84.3	3.8	78.4
9	2.0	88.9	1.6	90.9
10	1.8	89.9	1.4	92.1

is in accord with our previous results, since the older cultures in this case contained more actual vegetative mycelium. The difference in utilization is, however, greater than can be assigned to this cause alone.

A similar experiment was conducted to see if the presence of methylglucoside in the original medium would lead to a greater response. It was carried out under as nearly the same conditions as possible.

Table V shows that there is not so great a difference in the ability to utilize the glucoside before and after spore formation when the culture is first grown on a medium containing glucoside. This seems to indicate an adaptation of *Aspergillus niger* to

α -methylglucoside. Control flasks containing the sterile medium were carried along with these experiments. In every case there was a slight increase in rotation likely due to evaporation. This showed that there was no hydrolysis or molecular rearrangement of the glucoside due to standing at that temperature for a number of days.

Another attempt at adaptation of *Aspergillus niger* to α -methylglucoside was conducted in the following manner. A culture medium containing both sucrose and α -methylglucoside was prepared and distributed in a number of test-tubes, all of which were then sterilized. Inoculations were made successively from one tube to another as soon as the culture reached maturity. After cultivating the mold for nine generations on this medium the tenth transfer was made to 50 cc. of a medium containing

TABLE VI.

Age of culture.	Control.		Attempted adaptation.	
	Rotation of medium.	Decrease.	Rotation of medium.	Decrease.
days	$^{\circ}$ V.	per cent	$^{\circ}$ V.	per cent
0	17.8	0.0	17.8	0.0
6	17.0	4.4	16.8	5.6
6	17.0	4.4	17.0	4.4

glucoside but no sucrose. As a control, a parallel culture which had been propagated for the same number of generations on sucrose alone was transferred to another 50 cc. of the glucoside medium. The results are given in Table VI.

The extent of adaptation, if any, is so slight after nine successive generations of *Aspergillus niger* grown in the presence of glucoside as to be within the range of normal cultural variations.

SUMMARY.

Aspergillus niger grows very poorly on media containing α -methylglucoside as the only source of carbon, but readily on sucrose media in the presence of the glucoside. A vigorous culture transferred entire to the glucoside medium without sucrose may use up the glucoside more rapidly than a culture obtained by

direct inoculation of this medium with spores. If the original medium contained both sucrose and glucoside the latter disappears more rapidly from the second medium containing glucoside but no sucrose than when the original medium contained sucrose alone. Also there was a slight difference between the activity of cultures before and after spore formation.

A gradual cumulative adaptation through a number of generations cultivated on this substrate could not be demonstrated with any degree of certainty.



PROPERTIES OF THE NUCLEOTIDES OBTAINED FROM YEAST NUCLEIC ACID.

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PLATES 4 AND 5.

(Received for publication, March 1, 1920.)

The four nucleotides composing the molecule of yeast nucleic acid have now been isolated in crystalline form. Of these adenosinphosphoric acid was isolated by different methods by Jones and Kennedy¹ and by Levene.² The two substances seemed to differ from one another on the point of the crystal water.

Cytidinphosphoric acid also was obtained in two laboratories (Thannhauser and Dorfmueller³ and Levene⁴) and the two substances seemed to differ in their optical activity. Also the two samples isolated by the present writer showed minor differences in their rotatory power. The properties of guanosinphosphoric⁵ and uridinphosphoric acids⁶ had been given as found on substances as they were first obtained without further recrystallization.

The present work was undertaken for the sake of clearing up the above mentioned discrepancies and, further, for the sake of establishing the composition of the nucleotide as regards the crystal water, and for the sake of establishing the physical constants with greater rigor. In order to make the work possible, larger quantities of the crude material were required and were prepared. The results of the analysis of the carefully purified substance are as follows:

¹ Jones, W., and Kennedy, R. P., *J. Pharm. and Exp. Therap.*, 1919, xiii, 45.

² Levene, P. A., *J. Biol. Chem.*, 1919, xl, 415.

³ Thannhauser, S. J., and Dorfmueller, G., *Z. physiol. Chem.*, 1919, 104, 5.

⁴ Levene, P. A., *J. Biol. Chem.*, 1920, xli, 19.

⁵ Levene, P. A., *J. Biol. Chem.*, 1919, xl, 171.

⁶ Levene, P. A., *J. Biol. Chem.*, 1920, xli, 1.

Adenosinphosphoric acid (Fig. 1), according to Jones and Kennedy,¹ crystallizes with 1 molecule of crystal water. Levene, in his original work, found the air-dry substance anhydrous. The observation could not be repeated. Many samples have been analyzed since the first publication and all contained 1 molecule of crystal water. This was easily removed by drying under diminished pressure at the temperature of xylene vapor for 24 hours. The original sample was reanalyzed and again found practically anhydrous (1 per cent of water). In a sealed capillary tube the air-dry substance decomposed with effervescence at 195°C. (corrected).

Guanosinphosphoric acid (Fig. 2) crystallized in long needles of the appearance of the crystals of guanosin. It crystallized with 2 molecules of crystal water, which could be removed completely on drying under diminished pressure at the temperature of xylene vapor. In a sealed capillary tube the air-dry substance softened and became semitransparent at 175°C. and melted at 180°C. (corrected).

Uridinphosphoric acid (Fig. 3) was recrystallized out of methyl alcohol. The crystals formed slowly on evaporation of the alcohol. The larger crystals were often superimposed by deposits of smaller crystals, hence the photographic reproduction of the crystals was difficult. Besides, the yield of the material was rather small. For this reason it was decided to measure the optical rotation also of the monoammonium salt, which has good physical properties and a convenient solubility. The free acid crystallized in elongated prisms with pointed ends and melted in a sealed capillary tube at 198.5°C.

The monoammonium salt crystallized in prismatic needles which contained no crystal water. The air-dry substance heated in a sealed tube contracted and turned semitransparent at 200°C. (corrected), and decomposed at 242°C.

Cytidinphosphoric acid (Fig. 4) on repeated recrystallizations appeared in form of elongated plates which contained no crystal water. In a sealed capillary tube the air-dry substance decomposed with effervescence at 230–233°C.

Rotations.

The rotations were measured: (a) in aqueous solution, (b) in a solution of 10 per cent hydrochloric acid, (c) in a 5 per cent aqueous solution of ammonia, (d) in a 2 per cent aqueous solution of caustic soda, and (e) in a 5 per cent solution of the same.

Guanosin- and adenosinphosphoric acids showed a minimum levorotation in hydrochloric acid solution, which increased in water and in aqueous ammonia, and reached a maximum in an aqueous solution of sodium hydroxide.

Cytidinphosphoric acid showed a maximum dextrorotation in aqueous solution, successively decreasing in aqueous ammonia, hydrochloric acid, 2 per cent sodium hydroxide, and turned levorotary in 10 per cent sodium hydroxide.

Uridinphosphoric acid and its ammonium salt changed their optical rotation in the same direction as the preceding substance.

The changes in the optical rotation probably are the resultants of more than one factor. Among these the tautomeric changes in the basic radical of the substance possibly play an important part. Indeed, adenosin and uridin showed the same character of optical rotation as the corresponding nucleotides. On the other hand, the molecular rotation of the nucleoside and of the nucleotide are not identical.

EXPERIMENTAL.

Guanosinphosphoric Acid.—10 gm. of the crystalline material (No. 26) described in a previous communication⁵ were recrystallized out of water. One part of the substance was suspended in thirty parts of water and the water kept boiling until solution was completed. The solution was allowed to stand at room temperature (about 25°C.) over night. The yield of recrystallized material was 7.5 gm. (No. 291). It was planned to continue recrystallization until a constant optical rotation of the substance was attained. On the first recrystallization the specific rotation remained without change. No. 291 was recrystallized once more out of 200 cc. of water in the same manner as No. 26. The resulting material showed no change in its optical rotation. The air-dry substance heated in a sealed capillary tube contracted and turned semitransparent at 175°C. (corrected) and decomposed at 180°C.

486 Nucleotides from Yeast Nucleic Acid

0.1168 gm. of the air-dry substance on drying to constant weight under diminished pressure at the temperature of xylene vapor lost 0.1112 gm. in weight.

	Calculated for $C_{10}H_{14}N_2PO_8 + 2H_2O$.	Found.
	per cent	per cent
H ₂ O.....	9.47	9.67

The dry substance (No. 312) analyzed as follows:

0.1056 gm. of the substance gave 0.1312 gm. of CO₂ and 0.0168 gm. of H₂O.

0.1807 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 34.85 cc. of 0.1 N acid.

0.2710 gm. of the substance gave 0.0839 gm. of Mg₂P₂O₇.

	Calculated for $C_{10}H_{14}N_2PO_8$.	Found.
	per cent	per cent
C.....	33.05	33.89
H.....	3.89	3.90
N.....	19.22	19.25
P.....	8.55	8.62

Rotation of the substance in aqueous solution:

$$\text{No. 26} \quad [\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5^\circ$$

$$\text{No. 291} \quad [\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5^\circ$$

$$\text{No. 312} \quad [\alpha]_D^{25} = \frac{-0.16 \times 100}{1 \times 2} = -8.0^\circ$$

Rotation of the air-dry substance in 10 per cent hydrochloric acid:

$$\text{No. 312} \quad [\alpha]_D^{25} = \frac{+0.03 \times 100}{1 \times 2} = +1.5^\circ$$

Rotation of the air-dry substance in 5 per cent aqueous ammonia solution:

$$\text{No. 312} \quad [\alpha]_D^{25} = \frac{-0.88 \times 100}{1 \times 2} = -44.0^\circ$$

The rotation of the substance in 2 per cent aqueous caustic soda was as follows:

$$[\alpha]_D^{25} = \frac{-1.14 \times 100}{1 \times 2} = -57.0^\circ$$

In 5 per cent of the same:

$$[\alpha]_D^{25} = \frac{-1.30 \times 100}{1 \times 2} = -65.0^\circ$$

Adenosinphosphoric acid is the most insoluble of the four nucleotides. 23 gm. of the substance (No. 286) were suspended in 1 liter of hot water and the water was kept boiling for some time and since the substance did not dissolve readily dilute ammonia was added gradually until solution was completed. The hot solution was rendered slightly acid to litmus by means of acetic acid and allowed to stand at room temperature over night. A crystalline deposit (No. 282) formed.

The precipitate thus formed was recrystallized out of 600 cc. of water and allowed to crystallize over night. A crystalline deposit was formed. The yield was 7.5 gm. (No. 290). These were again recrystallized out of hot water. The yield was 5 gm. (No. 306). On heating in a sealed capillary tube the substance decomposed at 195°C. (corrected).

Analysis of the substance:

0.1325 gm. of the substance on drying under diminished pressure at the temperature of xylene vapor lost 0.0069 gm. of water.

	Calculated for $C_{10}H_{14}N_5PO_7 + H_2O$		Found.
	per cent		per cent
H ₂ O.....	4.90		5.20

Several samples were analyzed with the same result whereas the first two samples were analyzed for the anhydrous substance.

The dry substance (No. 306) analyzed as follows:

0.1256 gm. of the substance gave on combustion 0.1599 gm. of CO₂ and 0.0456 gm. of H₂O.

0.0948 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 13.56 cc. of 0.1 N acid.

0.2844 gm. of the substance gave 0.0913 gm. of Mg₂P₂O₇.

	Calculated for $C_{10}H_{14}N_5PO_7$		Found.
	per cent		per cent
C.....	34.57		34.71
H.....	4.07		4.06
N.....	20.17		20.13
P.....	8.94		9.04

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Rotation of the substance in 1 per cent aqueous solution in 2 dm. tube:

$$\text{No. 306} \quad [\alpha]_D^{20} = \frac{-0.81 \times 100}{2 \times 1} = -40.5^\circ$$

Since the substance is only little soluble in water the readings of the substances in process of their purification were taken in 5 per cent aqueous ammonia.

Rotation in 5 per cent aqueous ammonia solution:

$$\text{No. 282} \quad [\alpha]_D^{20} = \frac{-0.81 \times 100}{1 \times 2} = -40.5^\circ$$

$$\text{No. 290} \quad [\alpha]_D^{20} = \frac{-0.89 \times 100}{1 \times 2} = -44.5^\circ$$

$$\text{No. 306} \quad [\alpha]_D^{20} = \frac{-0.83 \times 100}{1 \times 2} = -41.5^\circ$$

The slight variation might be due to variation in the moisture of the substance.

Rotation in a solution of 10 per cent hydrochloric acid:

$$\text{No. 306} \quad [\alpha]_D^{20} = \frac{-0.76 \times 100}{1 \times 2} = -38.00^\circ$$

The rotation of the substance in 2 per cent aqueous caustic soda was as follows:

$$[\alpha]_D^{20} = \frac{-1.19 \times 100}{1 \times 2} = -59.5^\circ$$

In 5 per cent of the same:

$$[\alpha]_D^{20} = \frac{-1.32 \times 100}{1 \times 2} = -66.0^\circ$$

Cytidinphosphoric Acid.—The starting material was a mixture of cytidin- and adenosinphosphoric acid, prepared in the manner described in the previous paper. The original optical rotation was $[\alpha]_D^{20} = +25$. By recrystallization out of 50 per cent alcohol finally a substance was obtained with $[\alpha]_D^{20} = +40$. 10.0 gm. of this material (No. 270) were dissolved in 500 cc. of boiling water and to the solution 500.0 cc. of 99.8 per cent alcohol were added. Soon heavy crystals began to settle out and the crystallization was allowed to proceed 48 hours. The yield of the crystals was 7.5 gm. (No. 280). This material was then dissolved

400 cc. of boiling water and 400 cc. of 99.8 per cent alcohol were added to the solution. The crystallization proceeded as above. The yield of the crystals was 6.0 gm. (No. 285). These 6.0 gm. were dissolved in 350 cc. of boiling water and to the solution 150.0 cc. of 99.8 per cent alcohol were added. The yield of the final material (No. 289) was 5.0 gm. The crystal form is reproduced in Fig. 4.

The substance in sealed capillary tube decomposed with effervescence at 230–233°C. (corrected). (Heating slow.)

The analysis of the substance was as follows:

On drying under diminished pressure at the temperature of xylene vapor the substance lost 0.8 per cent in weight.

0.1123 gm. of the dry substance gave 0.1374 gm. of CO_2 and 0.0442 gm. of H_2O .

0.0992 gm. of the dry substance employed for Kjeldahl nitrogen estimation required for neutralization 9.23 cc. of 0.1 N acid.

0.2976 gm. of the substance gave 0.1014 gm. of $\text{Mg}_2\text{P}_2\text{O}_7$.

	Calculated for $\text{C}_9\text{H}_{14}\text{N}_2\text{P}_2\text{O}_8$	Found.
	per cent	per cent
C.....	33.42	33.36
H.....	4.37	4.41
N.....	13.00	13.03
P.....	9.61	9.50

The optical rotation was as follows:

In aqueous solution:

$$\text{No. 270} \quad [\alpha]_D^{20} = \frac{+ 0.80 \times 100}{1 \times 2} = + 40.0^\circ$$

$$\text{No. 285} \quad [\alpha]_D^{20} = \frac{+ 0.97 \times 100}{1 \times 2} = + 48.5^\circ$$

$$\text{No. 289} \quad [\alpha]_D^{20} = \frac{+ 0.95 \times 100}{1 \times 2} = + 47.5^\circ$$

In 10 per cent hydrochloric acid solution:

$$\text{No. 289} \quad [\alpha]_D^{20} = \frac{+ 0.52 \times 100}{1 \times 2} = + 26.0^\circ$$

In 5 per cent ammoniacal solution:

$$\text{No. 289} \quad [\alpha]_D^{20} = \frac{+ 0.89 \times 100}{1 \times 2} = + 44.5^\circ$$

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In 2 per cent sodium hydroxide solution:

$$\text{No. 289} \quad [\alpha]_D^{25} = \frac{+ 0.51 \times 100}{1 \times 2} = + 25.5^\circ$$

In 5 per cent solution of the same:

$$[\alpha]_D^{25} = \frac{+ 0.02 \times 100}{1 \times 2} = + 1.0^\circ$$

In 10 per cent solution of the same:

$$[\alpha]_D^{25} = \frac{- 0.42 \times 100}{1 \times 2} = - 21.0^\circ$$

Uridinphosphoric Acid.—The material described in a previous communication was dissolved in boiling methyl alcohol and the solution was allowed to stand in a desiccator over sulfuric acid. On standing, after a considerable part of the alcohol evaporated, heavy crystals settled out on the walls of the dish. The crystallization proceeded very slowly.

The substance melted in sealed tube with decomposition at M.P. = 198.5°C. (corrected).

The analysis of the substance was as follows:

0.1000 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 5.97 cc. of 0.1 N acid.

	Calculated for $C_8H_{12}N_2PO_8$	Found.
	per cent	per cent
N.....	8.64	8.36

The rotation of the substance (No. 343) was as follows:

In aqueous solution:

$$[\alpha]_D^{25} = \frac{+ 0.19 \times 100}{1 \times 2} = + 9.5^\circ$$

In 2 per cent solution of sodium hydroxide:

$$[\alpha]_D^{25} = \frac{+ 0.13 \times 100}{1 \times 2} = + 6.5^\circ$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{25} = \frac{- 0.30 \times 100}{1 \times 2} = - 15.0^\circ$$

Monoammonium Salt of Uridinphosphoric Acid.—The crystalline material described in a previous communication was dissolved in a minimum amount of water and to the solution 20 volumes of methyl alcohol were added. The solution was allowed to stand at room temperature. After several days on walls of the flask a sediment formed, which consisted of fine curved felt-forming needles (No. 254). In the mother liquor, on further standing, a third precipitate formed. The crystal form is reproduced in Fig. 3.

The substance when heated in a sealed capillary tube contracted and turned semitransparent at 200°C. (corrected) and decomposed with effervescence at 240°C.

The analysis of the substance was as follows:

0.1204 gm. of the substance gave on combustion 0.1417 gm. of CO₂ and 0.0514 gm. of H₂O.

0.1964 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 17.24 cc. of 0.1 N acid.

0.1964 gm. of the substance gave 0.0650 gm. of Mg₂P₂O₇.

	Calculated for C ₉ H ₁₂ N ₂ PO ₈ .	Found.
	per cent	per cent
C.....	31.66	32.09
H.....	4.73	4.77
N.....	12.32	12.30
P.....	9.58	9.15

The optical rotation of the substance was as follows:

In aqueous solution:

$$\text{No. 255} \quad [\alpha]_D^{20} = \frac{+ 0.21 \times 100}{1 \times 2} = + 10.5^\circ$$

In 10 per cent hydrochloric acid solution:

$$\text{No. 255} \quad [\alpha]_D^{20} = \frac{+ 0.05 \times 100}{1 \times 2} = + 2.5^\circ$$

In 5 per cent ammoniacal solution:

$$\text{No. 255} \quad [\alpha]_D^{20} = \frac{+ 0.28 \times 100}{1 \times 2} = + 14.0^\circ$$

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The rotation of the substance in 2 per cent aqueous sodium hydroxide was:

$$[\alpha]_D^{20} = \frac{+ 0.03 \times 100}{1 \times 2} = + 1.5^\circ$$

In 5 per cent solution of the same:

$$[\alpha]_D^{20} = \frac{- 0.32 \times 100}{1 \times 2} = - 16.0^\circ$$

In 10 per cent solution of the same:

$$[\alpha]_D^{20} = \frac{- 0.52 \times 100}{1 \times 2} = - 26.0^\circ$$

The optical rotation of adenosin was as follows:

In aqueous solution:

$$[\alpha]_D^{20} = \frac{- 1.20 \times 100}{1 \times 2} = - 60.0^\circ$$

In 10 per cent solution of hydrochloric acid:

$$[\alpha]_D^{20} = \frac{- 0.87 \times 100}{1 \times 2} = - 43.5^\circ$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{20} = \frac{- 1.37 \times 100}{1 \times 2} = - 68.5^\circ$$

The optical rotation of uridin was as follows:

In aqueous solution:

$$[\alpha]_D^{20} = \frac{+ 0.08 \times 100}{1 \times 2} = + 4.0^\circ$$

In 10 per cent solution of hydrochloric acid:

$$[\alpha]_D^{20} = \frac{+ 0.10 \times 100}{1 \times 2} = + 5.0^\circ$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{20} = \frac{- 0.12 \times 100}{1 \times 2} = - 6.0^\circ$$

EXPLANATION OF PLATES. .

PLATE 4.

- FIG. 1.** Crystals of adenosinphosphoric acid.
FIG. 2. Crystals of guanosinphosphoric acid.

PLATE 5.

- FIG. 3.** Crystals of monoammonium salt of uridinphosphoric acid.
FIG. 4. Crystals of cytidinphosphoric acid.



FIG. 1.

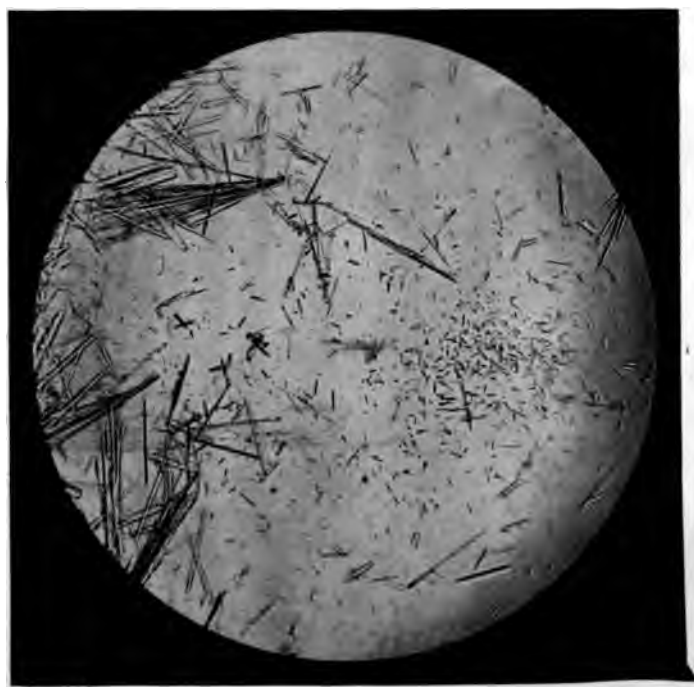


FIG. 2.

(Same. Nucleotides from yeast nucleic acid.)

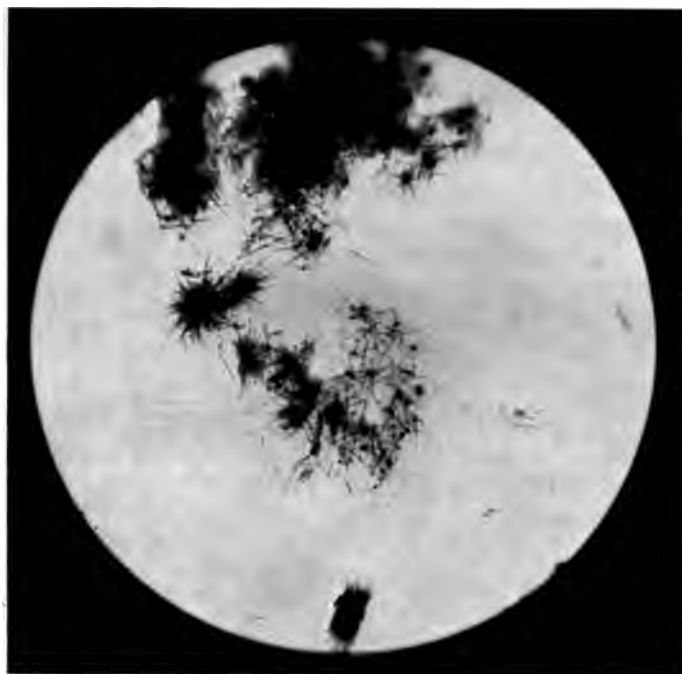


FIG. 3.



FIG. 4.

(Lewin: Nucleotides from yeast nucleic acid.)

RUTIN, THE FLAVONE PIGMENT OF *ESCHOLTZIA CALIFORNICA* CHAM.*

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PLATES 6 AND 7.

(Received for publication, February 7, 1920.)

The simple chemical relationship between the flavonol and anthocyanin series of plant pigments, suggested by Combes¹ and Everest,² but not proved until Willstätter and Mallison³ actually produced cyanidin from quercetin by reduction in acid solution, has led to considerable speculation as to the genetical and physiological interrelations of these compounds. There is some evidence that the anthocyanins are produced in the plant from the corresponding flavonols, and not by direct synthesis. Everest,⁴ for example, has shown that glucosides of the chemically related pair myricetin and delphinidin occur side by side in purple-black forms of *Viola*. Before far reaching conclusions are drawn, however, it will be necessary to isolate, or otherwise identify, the pigments of a large number of species. It goes without saying that the best material for this purpose will be afforded by species whose color varieties are capable of genetic analysis or by species in which the relations between flavone and anthocyanin are capable of experimental modification.

The genus *Escholtzia*, abundantly distributed in California, and common in cultivation, contains garden forms with yellow, golden yellow, pale yellow, white, carmine, and rose flowers. It

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¹ Combes, R., *Compt. rend. Acad.*, 1913, clvii, 1002, 1454.

² Everest, A. E., *Proc. Roy. Soc. London, Series B*, 1913-14, lxxxvii, 444.

³ Willstätter, R., and Mallison, H., *Sitzungsb. kais. Akad. Wissensch.*, 1914, 769.

⁴ Everest, A. E., *Proc. Roy. Soc. London, Series B*, 1917-18, xc, 251.

would provide ideal material for combined genetical and biochemical investigation providing the pigments concerned were present in large quantity and easily isolable. With this idea in mind, the writers obtained for preliminary work a large quantity of petals of wild *Escholtzia*, gathered by Mr. W. W. Wagener in the vicinity of Palo Alto, California. We have called the material *Escholtzia californica* Cham., using this specific name in the broad sense, for it was of course out of the question to observe close specific or varietal differences in gathering wild material of this polymorphic genus.

Preparation and Properties of Rutin.

The air-dried petals afforded an abundant yield of the glucoside rutin, quercetin glucoso-rhamnoside. They were first extracted for several days with ether, to remove fats, carotinoids, *etc.*, and then with ethyl alcohol. The alcoholic solution was evaporated to small bulk, poured into water, and the remaining alcohol boiled off. The crude rutin, which came down as a copious crystalline precipitate, was collected on a Buchner funnel, washed with water, dried, extracted with ether until no colored impurities were removed, and finally purified by recrystallization from a large volume of hot water. By this method 7.13 gm. of rutin (dried at 140°C.) were obtained from 150 gm. of air-dried petals.

According to Perkin and Everest,⁵ rutin "is said to melt above 190°." Schmidt⁶ gives 188–190°. Our preparation began to sinter at 186° and melted at 190–192°C. (uncorrected). The color of the anhydrous compound (dried at 150–160°), as ascertained by comparison with Ridgway's standards,⁷ was primrose-yellow; the streak was pale green-yellow. As obtained by crystallization from hot water *Escholtzia* rutin formed microscopically fine, very dense, acute-based tufts of silky crystals (Fig. 1).

Water of crystallization was determined by exposing a sample of the glucoside to a moist atmosphere under a bell jar until it

⁵ Perkin, A. G., and Everest, A. E., *The natural organic colouring matters*, London and New York, 1918, 197.

⁶ Schmidt, E., *Arch. Pharm.*, 1908, cexlvi, 214.

⁷ Ridgway, R., *Color standards and color nomenclature*, Washington, 1912.

came to constant weight. It was then dried at 160° for 12 hours. The loss of water from 2.2548 gm. of glucoside was 0.1868 gm., or 8.28 per cent, according satisfactorily with the 8.13 per cent calculated from $C_{27}H_{30}O_{16} \cdot 3H_2O$, the accepted formula of rutin.

Identification of Quercetin.

Hydrolysis resolves rutin into one molecule each of quercetin, rhamnose, and glucose. Our anhydrous preparation, boiled with approximately 5 per cent sulfuric acid, gave quercetin yields of 0.5354 gm. and 0.6177 gm. from samples weighing 1.0812 gm. and 1.2460 gm., respectively. These figures correspond to 49.51 and 49.57 per cent. Theory requires 49.51 per cent. The

TABLE I.

	Weight of sample.	CO ₂	H ₂ O	C	H	O
	gm.	gm.	gm.	per cent	per cent	per cent
Rutin.....	0.1645	0.3204	0.0766	53.12	5.22	41.66
	0.1810	0.3528	0.0840	53.15	5.20	41.65
Quercetin.....	0.1694	0.3709	0.0490	59.71	3.24	37.05
	0.1759	0.3854	0.0532	59.74	3.39	36.87
Penta-acetylquercetin.....	0.1220	0.2634	0.0423	58.88	3.89	37.23
	0.1106	0.2388	0.0386	58.88	3.91	37.21

crystalline quercetin (Fig. 2) was washed with cold water and dried at 140°C. It conformed in physical characteristics with quercetin from other sources. The crystals were citron-yellow; the streak light greenish yellow. In order to prevent decomposition of the material below the melting point, the bath (melted acid potassium sulfate) was heated to 300° before the sample for determination of melting point was introduced. Rosenthaler⁸ states that quercetin melts with partial decomposition at 310°. Our material darkened, but did not melt, between 300 and 305°. The melting point was not so sharp as might have been wished, but melting was complete at 310°. Wunderlich⁹ gives 305–310°.

⁸ Rosenthaler, L., *Der Nachweis organischer Verbindungen*, Stuttgart, 813.

⁹ Wunderlich, A., *Arch. Pharm.*, 1908, ccxvi, 224, 241, 256.

A satisfactory identification of the compound was secured, however, by acetylation. It gave penta-acetylquercetin, melting at 189–191°. Perkin and Hummel¹⁰ found 190–191°. Samples of the latter, weighing 0.7638 and 0.5704 gm., hydrolyzed by hydrochloric acid in glacial acetic acid, gave quercetin yields of 0.4512 and 0.3376 gm., corresponding to 59.07 and 59.18 per cent. Theory requires 58.98 per cent. Combustions were made of rutin and of the quercetin and acetylquercetin derived from it. The results, concordant and agreeing well with expectation, are given in Tables I and II.

When treated with sulfuric acid in boiling glacial acetic acid, quercetin forms a finely crystalline orange-vermilion acid addition product, $C_{16}H_{10}O_7 \cdot H_2SO_4$, from which the quercetin is easily regenerated by simple suspension in water. A sample of the

TABLE II.

	Rutin.		Quercetin.		Penta-acetylquercetin.	
	Found.	Expected.	Found.	Expected.	Found.	Expected.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C.....	53.14	53.10	59.73	59.59	58.88	58.59
H.....	5.21	4.95	3.32	3.34	3.90	3.90
O.....	41.65	41.95	36.95	37.07	37.22	37.51

sulfate weighing 0.2714 gm., dried at 100°, gave 0.2034 gm. of recovered quercetin, or 74.94 per cent. Theory requires 75.5 per cent.

The Sugars.

In view of the perfect agreement of the analytical results with the figures for rutin, complete identification required only the determination of the sugars resulting from hydrolysis. The literature of rutin and its synonyms, violaquercetrin, osyritrin, myrticolorin, *etc.*, shows that for many years the occurrence of glucose in the presence of rhamnose was overlooked, or *vice versa*. Following a modification of Perkin's procedure,¹¹ we separated pure glucosazone and rhamnosazone from the mixed osazones,

¹⁰ Perkin, A. G., and Hummel, J. J., *J. Chem. Soc.*, 1896, lxix, 1295.

¹¹ Perkin, A. G., *J. Chem. Soc.*, 1910, xcvii, 1776.

using the differential solubility of the compounds in acetone for the primary separation, following with recrystallization from 5 per cent pyridine in water and then from 20 per cent alcohol for final purification. In this manner rhamnosazone, melting at 181–182°, and glucosazone, melting at 205–207°, were obtained. Recrystallized from 20 per cent alcohol, under identical conditions, the crystal forms were characteristically different, as shown in Figs. 3 and 4. We were unable to distinguish two types of crystals in the mixture of osazones before fractionation by acetone, but after purification the glucosazone formed typical radiate groups of short, unbranched needles, and the rhamnosazone bifasciculate clusters of longer, more slender, branching needles.

Distribution of Rutin.

All the plants which have been reported to contain rutin have been critically examined in recent years either by Schmidt⁸ and Wunderlich⁹ or Perkin.¹¹ Adding *Escholtzia* to the list, the known distribution of rutin is now as follows:

Santalaceae; leaves of *Osyris compressa*,

Polygonaceae; entire herb, but chiefly the flowers, of *Fagopyrum esculentum*,

Papaveraceae; petals of *Escholtzia californica*,

Capparidaceae; flower buds of *Capparis spinosa*,

Leguminosae; flower buds of *Sophora japonica*,

Rutaceae; leaves of *Ruta graveolens*,

Violaceae; flowers of *Viola tricolor*,

Myrtaceae; leaves of *Eucalyptus macrorhyncha*,

Globulariaceae; leaves of *Globularia alypum*.

Only *Capparis* is doubtful, the rutin from this source differing from that of *Ruta*, etc. in sintering 10° below the usual temperature, regardless of every effort to purify the material completely. With the exception of *Globularia*, all the plants known to contain rutin fall within the subclass Archichlamideae of the Dicotyledones.

SUMMARY.

The petals of *Escholtzia californica* contain nearly 5 per cent of rutin (quercetin glucoso-rhamnoside). In view of the great range of flower colors in *Escholtzia*, from golden yellow to white, and from white to rose, this genus would appear to afford especially suitable material for study of the physiological and genetic relationships of the flavonol and anthocyanin pigments. It is hoped that the problems will interest workers who are advantageously located for carrying out both garden and laboratory studies.

Notwithstanding the brilliant work of Willstätter in showing the chemical relation of the anthocyanins and the flavonol pigments, it is quite true, as Wheldale¹² has said, that in order to prove their relation in nature it is necessary to know which flavone accompanies which anthocyanin in a considerable number of plants. It would conserve effort in solving the problem if the flavones were isolated and identified in all the plants in which Willstätter determined the anthocyanins, and, conversely, if those plants in which the yellow pigments are well known were studied with respect to the anthocyanins. That it will be difficult to work out the relation, and that it cannot be done except by collaboration between chemists and geneticists, is shown by the fact that Sutton's "Black Knight" pansy, a variety of *Viola tricolor*, a species well known for its great range of flower colors, has been shown by Everest to contain glucosides of the pair myricetin-delphinidin, whereas one would have expected from the well established occurrence of rutin in this species that the pair quercetin-cyanidin would have been the first to be detected. The share of the geneticist in the final elucidation of the pigment situation must be to provide the chemist with material of known factorial composition.

¹² Wheldale, M., The anthocyanin pigments of plants, Cambridge, 1916, 15.

EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. *Escholtzia* rutin, crystallized from hot water ($\times 90$).

FIG. 2. Quercetin from *Escholtzia* rutin, as obtained by hydrolysis of the rutin with boiling 5 per cent sulfuric acid ($\times 90$).

PLATE 7.

FIG. 3. Phenylglucosazone, separated from phenylrhamnosazone by acetone and purified by recrystallization. The crystals were obtained by cooling of a hot solution in 20 per cent alcohol. Under exactly the same conditions phenylrhamnosazone crystallized as in Fig. 4 ($\times 90$).

FIG. 4. Phenylrhamnosazone, crystallized from hot 20 per cent alcohol ($\times 90$).



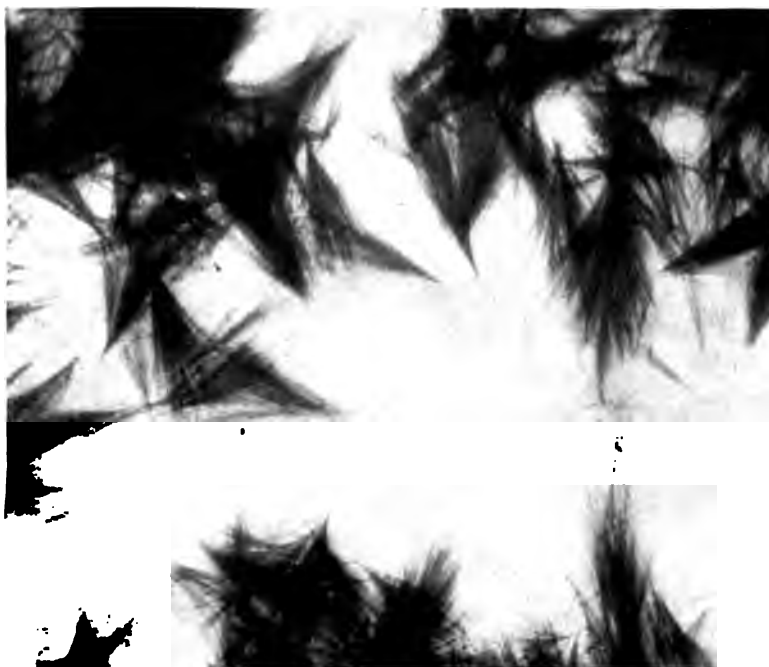


FIG. 1.



FIG. 2.

(Sando and Bartlett: Rutin.)

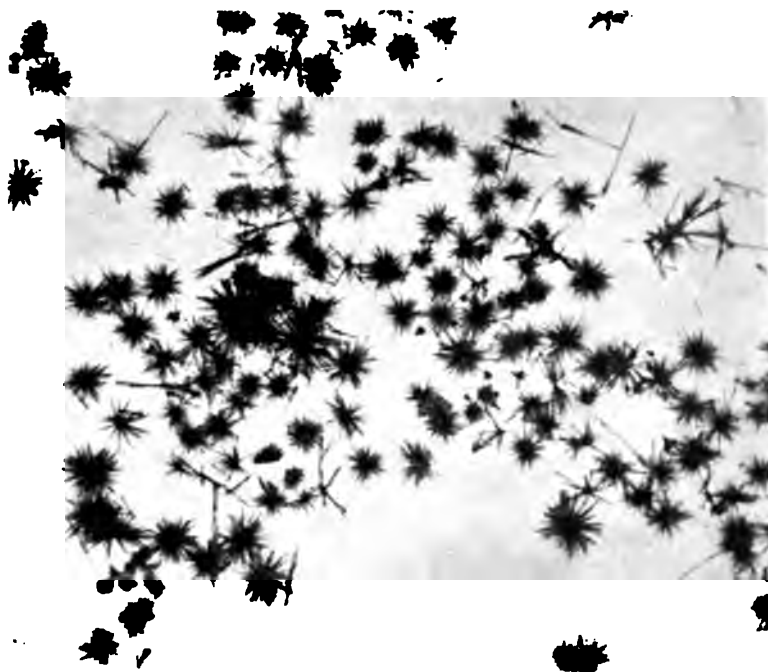


FIG. 3.



FIG. 4.

(Sando and Bartlett: *Rutin.*)

THE FORMATION OF ACETONE BODIES FOLLOWING ETHER ANESTHESIA AND THEIR RELATION TO THE PLASMA BICARBONATE.

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(Received for publication, February 10, 1920.)

It is a common observation that urine voided following ether anesthesia gives positive sodium nitroprusside and ferric chloride tests for acetone bodies; also, that as a result of the anesthetic there is a decrease in the CO_2 -combining power of the blood plasma. Because of this coincidence it has been believed by many that the formation of acetone bodies accounts for the decreased alkaline reserve. Some observations were recently made by Reimann and Bloom (1) which appeared to confirm this theory. They studied the CO_2 -combining power, together with the total blood acetone bodies, before and after ether anesthesia in a series of 60 patients and concluded that "blood acetone bodies account for 20 to 100 per cent of the bicarbonate fall observed, on an average for 60 per cent."

In the course of some work done to determine the frequency of acidosis in babies in a number of conditions, it was thought desirable to carry out some determinations of the blood acetone bodies, and for this purpose the method of Van Slyke and Fitz (2) was selected. The method is a gravimetric one and depends on the formation of a mercury-acetone complex which results from boiling mercuric sulfate, sulfuric acid, and potassium dichromate reagents in certain definite concentration with protein-free blood filtrate. Since certain substances beside acetone, including alcohol, give precipitates (2), it was necessary, if postoperative bloods were to be analyzed, to learn whether or not ether would also give a precipitate under the conditions of the determination.

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This proved to be the case, the precipitate forming only after boiling with the 5 per cent potassium dichromate reagent, which was reduced, giving a green color. This precipitate was very similar in appearance to the acetone-mercury compound, and like the latter gave a positive iodoform test (Lieben's). From these preliminary observations it seemed necessary to make a study of the acetone bodies before and after anesthesia on adult cases. The general plan was to observe the relation existing between the CO_2 -combining power of the blood and the β -hydroxybutyric acid, since this substance is in major part responsible for decrease in alkaline reserve when the decrease is due to a ketosis (3). It was also desired to observe the relative proportions of acetone and acetoacetic acid and β -hydroxybutyric acid, since it was thought that this would throw some light on the rôle of ether in the production of acetone bodies. The ether contained in the postoperative blood specimens was removed from the filtrate at the end of 30 minutes boiling with the reagents by passing a current of air through the hot liquid for 10 minutes, after the acetone precipitate had been filtered off. This was found by experiment to remove completely all the ether. After cooling, 160 cc. of the fluid were returned to the flask and 5 cc. of 5 per cent dichromate were added after boiling had commenced. The CO_2 -combining power was determined by the Van Slyke method (4).

The average of the preoperative values given in Table I, when expressed as total acetone bodies, is about 1 mg. per 100 cc. higher than that stated as the upper normal limit by Van Slyke and Fitz (2); the majority of those for acetoacetic acid and acetone (as acetone) and for β -hydroxybutyric acid are in agreement with those obtained by Marriott (5) for normal blood.¹ The average value for acetoacetic acid and acetone (as acetone) was 0.91 mg., that for β -acid 6.12 mg. per 100 cc. None of these patients was known to be suffering from a ketosis at the time these bloods were taken, the urines of all, except three which were not tested, being found negative to the ferric chloride and sodium nitro-

¹ It should be noted that β -hydroxybutyric acid values in Tables I, II, and III are expressed in terms of β -hydroxybutyric acid, while those given by Marriott are in terms of acetone. Conversion of the β -acid values to terms of acetone may be made by dividing by 2.36.

Patient.	Sex.	Age.	Preoperative.				Duration of ether administration.	Postoperative.				Remarks.				
			Urine	100 cc. of blood.				Urine.			100 cc. of blood.					
				Acetone bodies.*	Acetoacetic acid (as acetone).	β -hydroxybutyric acid.		CO ₂ -combining power.	mg.	mg.	per cent		Nature of collection.	Acetone bodies.	Acetoacetic acid (as acetone).	β -hydroxybutyric acid.
				mg.	mg.	per cent	min.		mg.	mg.	per cent					
1. J. R.	♂	25		2.4	8.04		21	1 voiding.			1.0	18.6				Subacute appendicitis. Appendectomy.
2. S. J.	♂	28	Negative.	0.0	3.55		30	1 "	Trace.*		1.2	3.52	47.5			Fistula repaired. Hemorrhoidectomy.
3. E. C.	♂	36	"	2.28	3.55	53.2	26	1 "	Faint trace.*		1.0	12.8	48.5			Hemorrhoidectomy.
4. W. D.	♂	37	"	1.0	4.28	69.2	40				1.0	5.53	59.8			Inoperable carcinoma of stomach.
5. E. C.	♀	32	"	0.6	6.62	51.3	45	24 hrs.	190 (total).		1.8	9.94	44.7			Femoral hernia.
6. E. P.	♂	74		0.4	2.36	53.0	40				0.75	0.0	44.7			Carcinoma of bladder.
7. P. O.	♂	55	Negative.	0.8	4.74		40				2.2	6.63	48.5			Hemorrhoidectomy.
8. M. B.	♀	36	"	0.2	24.6	54.0	70	24 hrs.	264 (total).		0.6	13.4	40.0			Carcinoma of breast.
9. J. D.	♀	77	"	1.4	1.51	58.9	110				2.0	14.15				Breast amputation for carcinoma.
10. W. C.	♂	50		0.8	7.0	62.4	43				0.4	16.6	51.9			Inoperable carcinoma of bladder.
11. M. M.	♀	27	Negative.	0.6	5.2	58.6	47	Hourly for 3 hrs.	See Table II.		0.6	3.55	50.0			Pericholecystic adhesions removed. Appendectomy.
12. M. F.	♂	29	"	0.48	2.0	55.1	77	Every 2 hrs. for 6 hrs.	See Table III.		1.0	4.68	46.2			Gastroenterostomy. Appendectomy. Removal of adhesions.

* Ferric chloride and sodium nitroprusside tests.

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prusside tests. The average duration of administration of ether for the series was 48 minutes. The average postoperative value for acetoacetic acid and acetone was 1.13 mg., and for β -acid 9.2 mg., per 100 cc. The average preoperative CO_2 -combining power was 57.3, postoperative, 48.6 volumes per cent. The average increase of acetoacetic acid and acetone was 0.22 mg., of β -acid 3.08 mg., per 100 cc. In several instances, but particularly in the case of M. B., there was apparently an actual decrease in the acetone bodies following anesthesia. As will be noted, the preoperative β -acid value for M. B. was exceptionally high; unfortunately, enough blood was not available or obtainable for a duplicate determination to check this result. In all cases the β -acid values were affected by the anesthetic to a greater extent than the acetoacetic acid and acetone values, the latter remaining more constant. The most significant rise in the β -acid value was seen in the case of J. D. where anesthesia was prolonged for 110 minutes, but in no case was the increase sufficient immediately after the anesthetic to become dangerous to the patient, as it is said that diabetics under good control show 10 to 40 mg. of acetone bodies (as acetone) per 100 cc. of blood (2). Unfortunately, sufficient blood was not obtained from J. D. following anesthesia for a determination of the CO_2 -combining power.

It would not only appear, from the figures in Table I, that the increased values for acetone bodies could have had little to do with the decreased bicarbonate, but it is also somewhat doubtful if the figures obtained on postoperative bloods in all cases represent merely the acetone bodies. It was suspected that, since the blood fat content is increased as a result of ether administration (6), this might become an interfering substance as its solution in the ether of the blood would permit it to escape through the filter during the removal of the precipitated proteins; and, since ether could readily be detected in the filtrate, a few experiments were carried out which confirmed this suspicion. At first, fat shaken with 25 cc. of water (in order to have the usual concentration of reagents) was boiled with the reagents including dichromate for $1\frac{1}{2}$ hours and a precipitate was recovered. Then some fat, of such quantity that, when added to 10 cc. of normal blood, it would approximately double its fat content, was thoroughly mixed with a 10 cc. portion of a blood specimen; and the

same amount of fat in solution in a small quantity of ether was thoroughly mixed with another 10 cc. portion of the same blood specimen. Determinations of total acetone bodies were then carried out in the usual manner, with the result that the specimen containing the ether yielded more than double the amount of precipitate obtained from the other. Since ether itself forms a precipitate when boiled with the reagents in the presence of dichromate, and since in this experiment the ether was not removed by aeration as previously described, the experiment was repeated, adding the same amount of fat, the fluid being aerated after 30 minutes boiling without dichromate to precipitate the preformed acetoacetic acid and acetone present in the blood. From the specimen to which fat alone was added, no increase in precipitate was obtained over that yielded by the blood alone, the figures being nearly identical. The precipitate from that to which both fat and ether were added, however, was over twelve times as great.

Following this work two series of determinations of acetone bodies, CO_2 -combining power, and total fat were made on the bloods of two patients before and after ether anesthesia. The fat determinations were carried out by Bloor's nephelometric method (7). From the first patient blood and urine specimens were collected simultaneously at hourly intervals for 3 hours, and from the second at 2 hour intervals for 6 hours. The urines were likewise analyzed for acetone bodies. The results are expressed in Tables II and III.

The figures in Table II express rather clearly the time necessary for any significant increase in concentration of blood acetone bodies. At 3.00 p.m., immediately after the cessation of 47 minutes of ether administration, there was no increase. At 4.00 p.m. there was apparently an increase in preformed acetoacetic acid and acetone, but still no increase in the β -acid. At 5.00 and at 6.00 p.m., however, the β -acid determinations showed a marked and distinct increase, rising to 6.63 and 10.4 mg., the acetoacetic acid and acetone value remaining about constant around 1 mg. This increase is confirmed by the increased output in the urine where there was a gradual increase for each hourly period coincident with the increased blood concentration. (In examining the table it should be noted that the first urine collection, at

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4.00 p.m., was for a period of 2 hours, the bladder being emptied at 2.00, and no collection being made at 3.00.)

In connection with the results of these determinations, it is now interesting to note what happened to the plasma CO_2 -combining powers for the same periods. Before anesthesia the figure obtained was 58.6; immediately after, it had fallen to 50.0; one hour after this, it had risen slightly to 52.8, and at the end of another hour it again had decreased to 50.0, coincident with an

TABLE II.

Determination of Acetone Bodies, CO_2 -Combining Power, and Total Fat.

M. M., ♀, aged 27. Pericholecystic adhesions removed. Appendectomy.

Time.	Urine.			100 cc. of blood.				Remarks.
	Volume.	Acetoacetic acid and acetone (as acetone).	β -hydroxybutyric acid.	Acetoacetic acid and acetone (as acetone).	β -hydroxybutyric acid.	Total fat.	CO_2 -combining power.	
	cc.	mg.	mg.	mg.	mg.	gm.	per cent	
2.00 p.m.		Negative.*		0.6	5.2	1.39	58.6	Bladder emptied.
2.13 "								Ether anesthesia begun.
3.00 "				0.6	3.55	1.51	50.0	Administration of ether ended.
4.00 "	57	1.37	2.87	1.2	2.84	1.47	52.8	
5.00 "	22	0.88	2.7	1.0	6.63	2.11	50.0	Slight hemolysis.
6.00 "	26	0.93	6.27	1.4	10.4	2.02	54.8	

* Ferric chloride and sodium nitroprusside tests.

Each of the postoperative blood specimens gave an odor of ether. Postoperative urines were withdrawn by catheter.

increase in β -acid from 2.84 mg. for the hour previous to 6.63 mg. It was thought, however, that this slight decrease was probably due to a slight hemolysis which was present in this specimen, but was not present in the others. The final determination is most significant, however, since there was a distinct increase in CO_2 -combining power, beyond any possible limit of error, to 54.8 coincident with an increase in β -acid from 6.63 to 10.4 mg.

Figures in Table III are in harmony with those in Table II, though they are not quite so striking. Urine figures show the same gradual increase. (The 3.45 p.m. collection of 243 cc. was for 1½ hours.) Unfortunately three of the blood acetone body determinations were accidentally spoiled. As a result of the anesthetic, the CO₂-combining power decreased from 55.1 to 46.2, coincident with an increase in the figures for acetoacetic acid and acetone from 0.48 to 1.0 mg., and for β -acid from 2.0 to

TABLE III.

Determination of Acetone Bodies, CO₂-Combining Power, and Total Fat.

M. P., ♂, aged 29. Posterior gastroenterostomy. Separation of adhesions. Appendectomy.

Time.	Urine.			100 cc. of blood.				Remarks.
	Volume.	Acetoacetic acid and acetone (as acetone).	β -hydroxybutyric acid.	Acetoacetic acid and acetone (as acetone).	β -hydroxybutyric acid.	Total fat.	CO ₂ -combining power.	
	cc.	mg.	mg.	mg.	mg.	gm.	per cent	
2.00 p.m.		Negative.*		0.48	2.0	0.653	55.1	Bladder emptied.
3.17 "								Ether anesthesia begun.
3.45 "	243	0.0	9.77	1.0	4.64	0.629	46.2	Administration of ether ended.
5.45 "	40	1.14	9.25		7.04	0.707	51.0	
7.45 "	33	2.18	6.64	0.6	6.04	0.725	51.0	
9.45 "	50	2.84	11.0			0.666	50.8	

* Ferric chloride and sodium nitroprusside tests.

Postoperative urines were withdrawn by catheter.

4.64 mg. The most significant feature, however, is the increase in CO₂-combining power from 46.2 to 51.0 during the next period of 2 hours coincident with an increase in β -acid from 4.64 to 7.04 mg.

Blood fat determinations were carried out in the last two cases because it was thought that, since traces of fat may escape through the filter during the removal of the proteins and appear in the filtrate, this might account in part for the increased value obtained

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following ether anesthesia. Very probably this is true, and many figures in Table I are undoubtedly higher than they should be, for the reason that the blood withdrawn immediately after anesthesia contained relatively large amounts of ether. This could not be aerated off before precipitation of the blood proteins or before boiling the filtrate with the reagents because of the probability of volatilizing the preformed acetone at the same time (8). The β -acid values expressed in Tables II and III, however, show definite, true increases in this substance, because the fat values remained constant while the β -acid values increased; the ether was becoming less concentrated in the later specimens; and, most important, corresponding increases in excretion were found in the urine. It would appear, therefore, from the results as a whole, that the blood acetone bodies could have had little to do with the decreased plasma bicarbonate after ether anesthesia. This decrease was undoubtedly due to other factors (9).

EXPERIMENTAL.

Interfering Substances.

1. *Ether*.—Ether was boiled with the reagents to determine whether or not a precipitate was formed from it. A precipitate formed only after the addition of 5 cc. of 5 per cent dichromate, the fluid becoming green in color. It was observed in this experiment that some ether was being lost through the reflux condenser.

2. *Fat*.—(A) Neutral olive oil was boiled with the reagents in usual concentration to determine whether or not this would give a precipitate, with the result that a precipitate formed in considerable quantity after dichromate was added. This was freed from excess fat by extraction with alcohol and ether.

(B) 60 mg. of neutral olive oil were thoroughly mixed with a 10 cc. portion of a blood specimen. A determination for total acetone bodies was then carried out on this specimen with the result that, when calculated per 100 cc. of blood, a value of 6.6 mg. was obtained. At the same time a solution of 60 mg. of olive oil in a little ether (about 1 or 2 cc.) was thoroughly mixed with another 10 cc. portion of the same blood. A determination for total acetone bodies was then carried out with the result that 15.8 mg. (as acetone) were obtained (calculated per 100 cc. of blood).

(C) 10 cc. of a blood specimen were analyzed for total acetone bodies in the usual manner. The result was 0.59 mg. as acetone per 100 cc. of blood. To another 10 cc. portion of the same blood specimen 60 mg. of olive oil (about equal to the normal fat content of 10 cc. of blood) were added, and an analysis was made as with the first portion. 0.51 mg. per 100 cc. of blood was obtained. To a third portion of the same blood specimen were added 60 mg. of olive oil in solution in a small amount of ether, and the whole was thoroughly mixed. An analysis was then made for total acetone bodies, the filtrate first being boiled with the H_2SO_4 and HgSO_4 reagents, but without dichromate for 30 minutes, to precipitate the preformed acetone. The hot liquid was then aerated for 10 minutes to remove the ether; 5 cc. of 5 per cent dichromate were added after boiling had commenced, and after boiling had continued for $1\frac{1}{2}$ hours longer the determination was completed. The result was 7.42 mg., as acetone, calculated per 100 cc. of blood, over twelve times the result obtained from analysis of the blood alone.

3. *Removal versus Non-Removal of Ether from Blood Filtrate.*—Total acetone bodies were determined on a specimen of blood, withdrawn immediately after ether anesthesia, having a strong odor of ether. The ether was removed by aeration after the first 30 minutes of boiling, as previously described. The result was 9.4 mg., as acetone, per 100 cc. of blood. A simultaneous determination of total acetone bodies was made on the same blood, no attempt being made to remove the ether. 10.0 mg., as acetone, per 100 cc. of blood were obtained.

4. *Glycerol.*—A few drops of glycerol, weighing exactly 129 mg., were placed in a 500 cc. Erlenmeyer flask with 25 cc. of water. Procedure was then carried out as for total acetone bodies. Upon the addition of the dichromate this was immediately reduced giving a green color. A precipitate of 462.2 mg. was formed which was only slightly soluble in HCl and gave a positive Lieben's, but a negative Gunning's, iodoform test.

5. *Oleic Acid.*—Oleic acid was treated in the same manner as the glycerol, 115.6 mg. forming 12.4 mg. of precipitate with the reagents.

Tests of the reagents showed that these alone formed no precipitate. No blank determinations were made on any of the blood or urine filtrates.

SUMMARY.

Observations of the acetoacetic acid and acetone (expressed as acetone) and the β -hydroxybutyric acid of the blood were made before and after ether anesthesia on twelve patients by the method of Van Slyke and Fitz, and the CO_2 -combining powers before and after anesthesia on eight of these patients by the Van Slyke method. A series of blood and urine specimens from each of two of these patients was also withdrawn over a number of hours following ether anesthesia, the blood, in addition to the above mentioned determinations, being examined for total fats by Bloor's method, and the urine for acetoacetic acid and acetone (as acetone) and β -hydroxybutyric acid by Van Slyke's method. A number of experiments were carried out to determine sources of error in analyzing blood containing ether. Removal of ether from blood filtrates was accomplished by aeration of the mixed filtrate and reagents after 30 minutes boiling to precipitate the preformed acetone and acetoacetic acid.

Contrary to the results of Reimann and Bloom, it was found that the blood concentration of acetone bodies was but little affected during the period of anesthesia, which was, on the average, a period of 48 minutes. It was found in the two cases examined over a longer period that there was an increase in these substances a few hours later, a finding which was confirmed by their increased output in the urine. The CO_2 -combining powers, however, increased even during increase of β -hydroxybutyric acid.

The results obtained for acetone bodies on bloods taken previous to anesthesia were, on the average, lower than those reported by Reimann and Bloom, the majority agreeing with the figures given by Van Slyke and Fitz, and also by Marriott, for normal blood. It is possible that Reimann and Bloom's high results find explanation in occasional failure to filter off their precipitate soon after the period of boiling was finished. Van Slyke and Fitz (10) have, since the appearance of Reimann and Bloom's paper, pointed out that this precaution is necessary.

Experimental data indicate that fat in the presence of ether and ether itself may interfere with the accuracy of the method, since both may form a precipitate with the reagents. It could not be shown from analyses on postoperative bloods that ether

directly effected much error, but through its ability to penetrate the filter it apparently carried with it some fat in solution causing an increase in precipitate. Glycerol formed a comparatively large precipitate with the reagents, oleic acid a much smaller precipitate.

CONCLUSIONS.

Acetone bodies were not formed promptly enough during ether anesthesia in the cases reported to account for the decreased plasma bicarbonate.

Analyses reported for β -hydroxybutyric acid on postoperative bloods may in some instances have been too high due to an error introduced as a result of the ether content.

The author wishes to thank Professor Ludwig Kast, of the Department of Medicine, for permission to engage in, and approval of, this work; Professor Victor C. Myers, of the Department of Laboratories, for helpful advice and suggestions; and Doctor C. H. Whiting, of the Department of Surgery, for his kind cooperation and assistance in attending to the collections of blood and urine specimens.

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MILK AS A SOURCE OF WATER-SOLUBLE VITAMINE. II.*

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Early in the course of our nutrition experiments upon rats¹ we learned that both milk—dried milk powder was used—and what we termed “protein-free milk” prepared from it furnished something without which the animals could not grow satisfactorily when they were kept on “synthetic” diets consisting of mixtures of more or less purified isolated food substances. Subsequently Hopkins² published striking experiments showing that fresh milk, used in quantities surprisingly small when compared to the amounts which we had incorporated into our successful rat diets, also was effective in securing growth on the so called synthetic food mixtures consisting of purified proteins, fat, carbohydrates, and salts. Experience had shown us the necessity of incorporating as much as 28 per cent of our protein-free milk into our food mixture in order to secure adequate growth. The actual milk solids furnished by some of the quantities of fresh milk which Hopkins used effectively amounted “to no more than from 1 to 3 or 4% of the whole food eaten.” The calorific value of Hopkins’ foods was comparable with that fed by us.

Since these pioneer experiments our knowledge of the significance of those nutrition-promoting substances now commonly

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

¹ Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911.

² Hopkins, F. G., *J. Physiol.*, 1912, xlv, 425.

designated as vitamines has been largely augmented. Bearing this in mind we undertook further investigations in the hope of explaining the apparent discrepancies between our earlier results and those of Hopkins as to the minimal amount of milk needed to supply an adequate quota of the water-soluble vitamine. The entire subject has already been reviewed in some detail by us.³

Attempting to duplicate the results of Hopkins we fed diets consisting of casein or edestin, starch, a salt mixture, lard, and butter fat along with fresh milk offered in varying quantities. In contradiction to some of Hopkins' results we found, under the conditions of our investigation, that 2 cc. of milk per day rarely sufficed to enable rats, on the diets mentioned, to make more than very slight gains in weight. Many of the animals were barely maintained when such small quantities furnished the sole source of water-soluble vitamine. Not until at least 16 cc. of fresh milk per day were supplied along with the food mixture was anything approaching a normal rate of growth secured. Even this amount sometimes failed.

The large quantity of fresh milk thus indicated as necessary to supply water-soluble vitamine is equivalent, broadly speaking, to the quantities of protein-free milk which were found necessary, in our earlier experiments, to secure adequate growth with the synthetic dietaries, even when due attention was paid to the calorific make-up of the food, the quality and content of its protein, and the supply of fat-soluble vitamine. As was pointed out in the previous report of our investigation we tested the possibility that pasteurization of milk, as it is conducted preliminary to the distribution of most city milk supplies, might affect the vitamine content of the milk employed by us. The use of unpasteurized milk of known origin did not improve our results; the smaller quantities—less than 16 cc.—still proved inadequate to promote growth at a maximum rate.

To another aspect of this question we have referred as follows:³

"That the deficiency of diets containing the lesser amounts of milk involves the vitamine factor is rendered more than probable by the fact that these comparatively small additions of yeast, the highly efficient growth-promoting power of which we have discussed elsewhere, sufficed

³ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 537.

to render the previously inadequate ration satisfactory for growth. As a rule the most significant outcome of the yeast additions (which were fed apart from the rest of the food and therefore could not have altered its flavor) was a larger food intake."

Our earlier investigations with fresh milk were carried out during the winter season when the cows in this region are deprived of green pasture and are stall-fed. It seemed not impossible, therefore, that the relatively large quantity of milk necessitated as a source of water-soluble vitamins in those experiments might be associated with quality of milk inferior from the vitamin standpoint, owing to the winter diet of the cows. Other investigators have of late intimated that the content of the milk in various nutrition-promoting factors may be markedly altered by the character of the diet from which the various vitamins are assumed to be derived. Our own investigations have taught us that the grasses and other green foods, which cattle are likely to obtain in greater abundance during the summer season, are rich in vitamins.⁴

Consequently we have undertaken a further series of experiments on rats which were supplied with a diet presumably adequate in every respect except for the absence of the water-soluble vitamin.⁵ The food mixtures consisted of:

	<i>per cent</i>
Casein.....	18
Salt mixture*.....	4
Starch.....	49
Butter fat.....	9
Lard.....	20

* The composition of the salt mixture used is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

In addition to this mixture, which was fed *ad libitum*, the rats received daily, in measured portions in a separate container,

⁴ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187; xxxix, 29.

⁵ In numerous experiments with such a food mixture we have demonstrated that the addition of 0.2 gm. of dried brewery yeast fed apart from the ration suffices to induce the animals to eat sufficient food and promote growth, whereas without the yeast addendum their food intake gradually declines and they die within a period of 40 to 80 days (Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35).

varying quantities of unpasteurized milk obtained fresh from cows known to be feeding in open pasture. Until within the last month of our experiments the five cows were kept all day in the pasture. During the last month, owing to the lateness of the season, the available pasturage was small. Night and morning the cows were fed with rations of corn gluten and wheat bran together with hay and corn stalks. This method of feeding is the one usually employed in this region where milch cows are pasture-fed during the summer.

The outcome has been comparable with the experiments earlier published, as the appended charts show. Thus with additions of 2 cc. of summer milk (Rats 5904, 5911, 5891, Chart I), fed during a period of 37 or more days, no permanent gains were secured. Additions of 5 cc. (see also Rats 5944, 5943, 5947, Chart II) invariably produced better, though by no means adequate, growth; nor was the latter usually obtained with daily additions of 10 cc. of summer milk. Whenever the vitamine supplement in the form of milk was still further increased, improvement in the rate of growth occurred. Of course, the rats ate more in these cases. The weekly food intakes, not including that furnished by the milk solids, are shown in the charts.

The inferiority of even 15 cc. of the fresh, unpasteurized summer milk as a source of water-soluble vitamine, in contrast with 0.2 gm. of dried brewery yeast, is indicated by the more rapid gains made by all the animals thus tested when the yeast addendum replaced the milk. In our previous report we have indicated that this is not due to the large volume of fluid intake, represented by 15 cc. of milk, preventing the ingestion of a sufficient amount of the basal diet; for in the experiments there recorded, when yeast was furnished in addition to both the large volume of milk and the basal diet, increased intakes of food with additional gains in weight followed.

When the experiments were *started* with 10 cc. of the summer milk, there was no greater advantage (see Chart III, Rats 6096, 6097, 6101); and even 15 cc. of milk fed from the start (Rats 6093, 6098, 6092, Chart III) barely sufficed as a source of water-soluble vitamine to promote growth at a normal rate. Here again an inspection of the charts shows the superiority of 0.2 gm. of dried yeast, in contrast with the large volumes of milk.

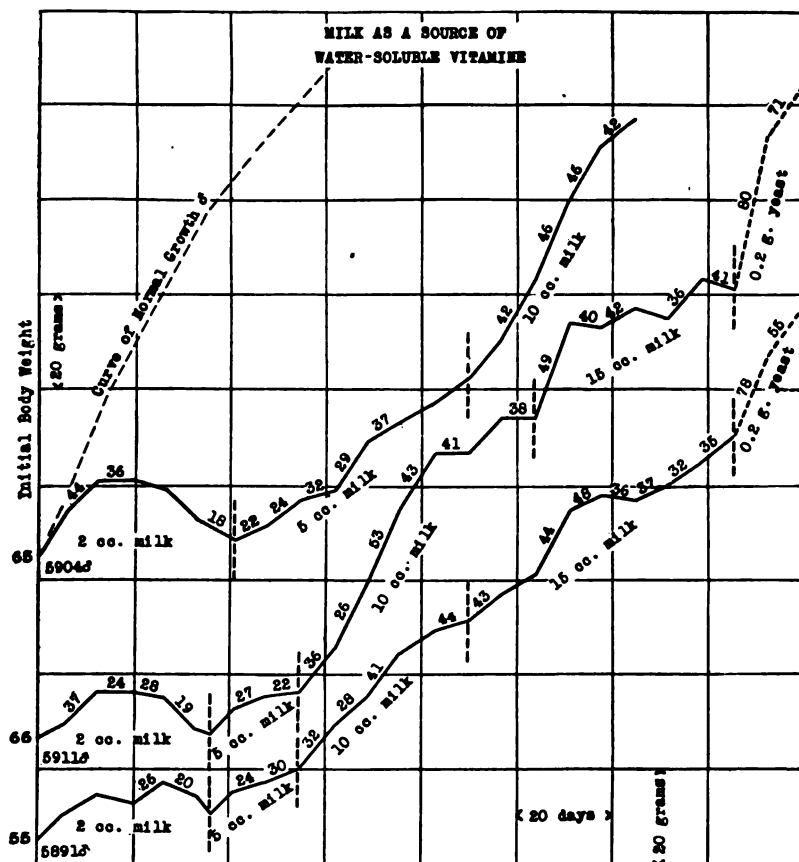


CHART I. Showing the effect upon growing white rats of increasing quantities of fresh unpasteurized summer cow's milk, fed in addition to and apart from a standard diet devoid of water-soluble vitamine. The composition of the food mixture is given on page 517. The weekly food intakes, expressed in gm. (exclusive of the milk solids), are indicated, where available, on the curve of growth. The interrupted line (Rats 5891 and 5911) indicates the superiority of small quantities of brewers' yeast in comparison with large volumes of milk as a source of water-soluble vitamine, and also the adequacy of the ration apart from its content of the latter.

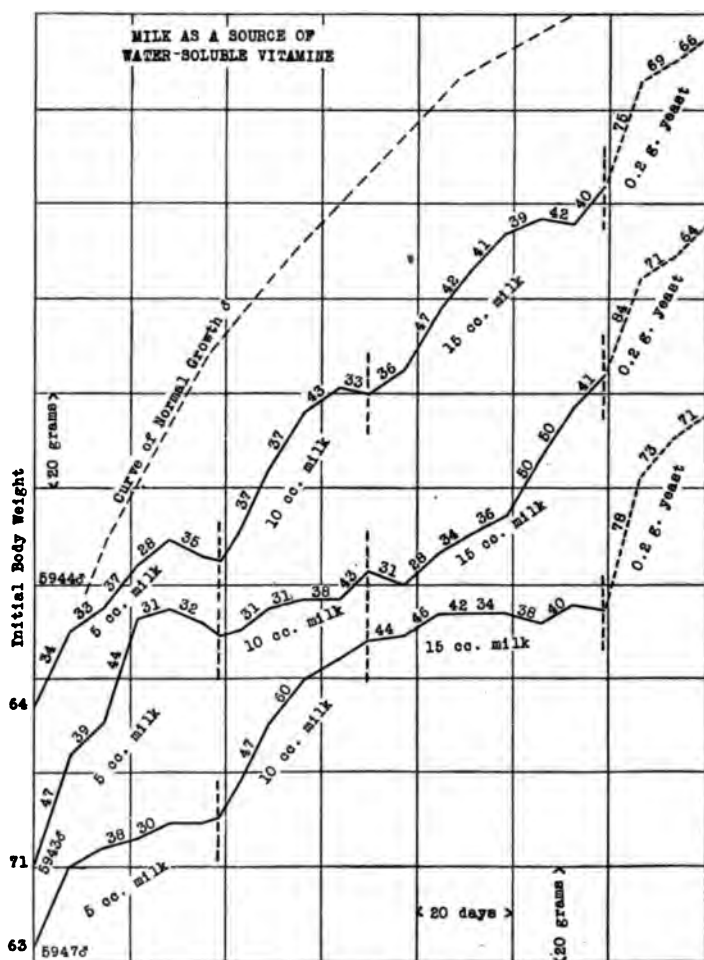


CHART II. Supplementing Chart I in showing the effect upon growing white rats of increasing quantities of fresh unpasteurized summer cow's milk, fed in addition to and apart from a standard diet devoid of water-soluble vitamine. The composition of the food mixture is given on page 517. The weekly food intakes, expressed in gm. (exclusive of the milk solids), are indicated, where available, on the curve of growth. The interrupted line (Rats 5944, 5943, 5947) indicates the superiority of small quantities of brewers' yeast in comparison with large volumes of milk as a source of water-soluble vitamine, and also the adequacy of the ration apart from its content of the latter.

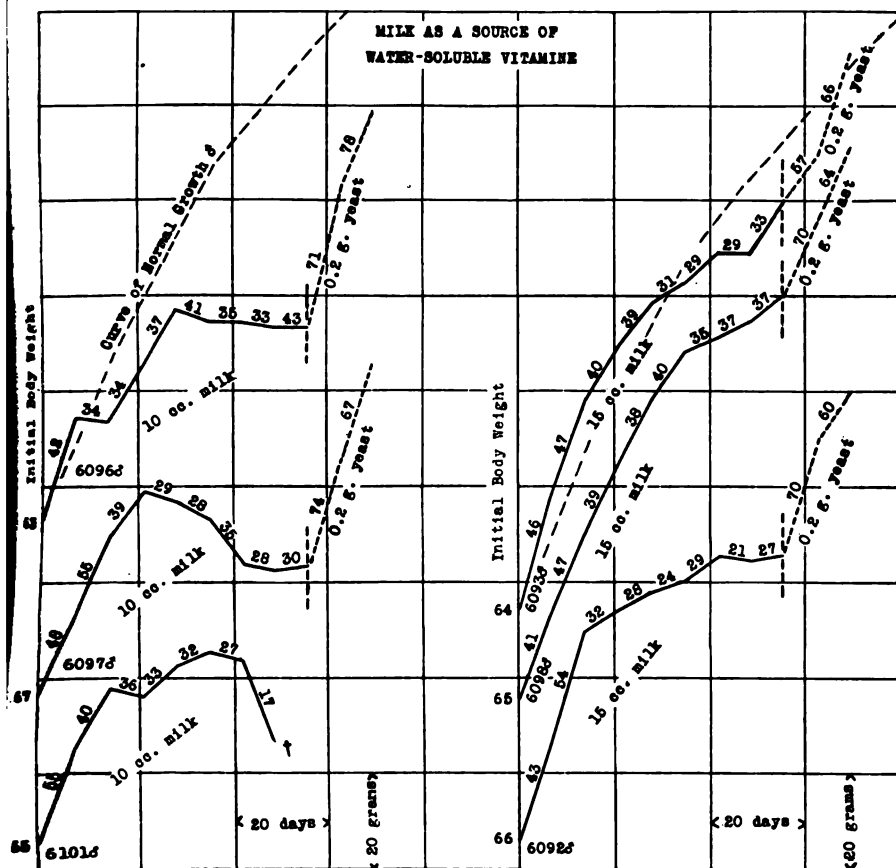


CHART III. Showing the failure of 10 cc. of fresh unpasteurized summer cow's milk (Rats 6096, 6097, 6101), fed apart from and in addition to a ration adequate except in respect to the lack of water-soluble vitamin, to promote growth at a normal rate. The weekly food intakes, expressed in gm. (exclusive of the milk solids), are indicated, where available, on the curve of growth. The prompt increase in food intake and growth response (shown by the interrupted line) when milk was replaced by small daily doses of brewers' yeast indicate that the previous failures were associated with the lack of water-soluble vitamin.

When 15 cc. of the same milk (Rats 6093, 6098, 6092) supplied the water-soluble vitamin better growth responses were obtained; but not so good as those secured by the feeding of 0.2 gm. of dried yeast.

In the original experiments of Hopkins the food was reported to have the following composition:²

	Pure casein mixture. <i>per cent</i>	"Protene" mixture. <i>per cent</i>
Protein.....	22.0	21.3
Starch.....	42.0	42.0
Cane Sugar.....	21.0	21.0
Lard.....	12.4	12.4
Salts*.....	2.6	3.3

* "The salts added were obtained by incinerating the normal laboratory food on which the rats had been kept when not under experiment, and consisted of equal parts of the ash of oats and dog-biscuits."

It was, of course, possible that the ash of oats and dog biscuits there used might contain some unsuspected inorganic ingredients which would account for the remarkable gains claimed by Hopkins for some of the animals to which the small quantities of milk were supplied. We therefore imitated the diets used by him as closely as the preparation of ash from a product of such uncertain composition as dog bread would permit. These food mixtures consisted of:

	<i>per cent</i>
Casein.....	18.0
Salts*.....	4.5
Starch.....	50.5
Butter fat.....	9.0
Lard.....	18.0

* The salts consisted of equal parts of the ash of dog bread and the ash of whole oats.

The outcome was in harmony with all our experience in showing that even additions of 10 cc. of fresh milk per day were insufficient to effect a food intake adequate for growth at a normal rate. Chart IV, showing the results in graphic form, also includes the growth curves of rats fed on diets containing our own often described salt mixture. The milk in the two series was obtained from the same source, so that the experiments are strictly comparable. The outcomes are not essentially different.

Recent studies of the antiscorbutic value of cow's milk⁶ have indicated that on this score it must be classed as less valuable than many of the raw fruits and vegetables. Whereas quantities of the latter—less than 10 gm. daily—will prevent scurvy in guinea pigs upon a diet otherwise devoid of antiscorbutic material, 100 to 150 cc. daily of raw cow's milk are required for this species, according to Barnes and Hume; while monkeys require larger quantities. Similarly, relatively large

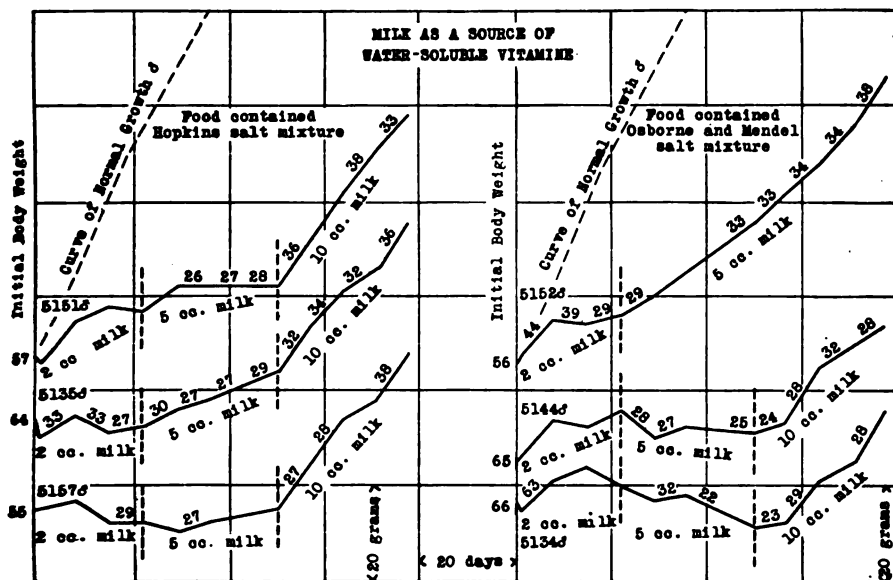


CHART IV. Showing that rats grow at approximately the same rate whether they receive a salt mixture similar to that used by Hopkins or the salt mixture used by ourselves, when like quantities of milk supply all the water-soluble vitamine. The weekly food intakes, expressed in gm. (exclusive of the milk solids), are indicated, where available, on the curve of growth.

quantities of milk are required to produce the increased intake of food and improved rate of growth, which are readily secured by very small quantities of many (dried) green vegetables. The consequences of this relative poverty of milk in water-soluble vitamine for the artificial feeding of infants have already been referred to by us.

⁶ Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425. Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131; *Lancet*, 1918, i, 1. Barnes, R. E., and Hume, E. M., *Lancet*, 1919, ii, 323.



THE EFFECT OF INTRAVENOUS INJECTIONS OF ACTIVE DEPOSIT OF RADIUM ON METABOLISM IN THE DOG.

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There have been few experiments reported on the effect of radium on normal metabolism. Berg and Welker (1) have studied the effect of radium salts upon the metabolism of dogs. The doses employed were very small, but these investigators concluded that ingestion of radium *per os* was followed by a stimulation of the catabolic processes as indicated by a slightly increased output of nitrogen in the urine. An increased volume of urine was also noted.

In experiments where radium salt is given, it is obvious that one cannot be certain whether the effects noted are due to the element radium or the rays emitted by the element.

The present work has been planned to study the effect of the active rays upon the general metabolism of the dog. Solutions of sodium chloride which contained active deposit from radium emanation were used. To prepare the solutions, sodium chloride is first dried and packed into a bulb and left in contact with radium emanation for 3 or 4 hours.¹ The emanation is then pumped off and the salt dissolved in water in such concentration as to give a solution isotonic with the blood. The solution is drawn into a syringe and the amount of activity determined by a γ -ray instrument. After the injection, the radioactivity remaining in the syringe is again determined and the amount injected computed by difference. The radioactivity is expressed in millicuries. It should be noted that the number of millicuries

¹ The preparation of the radioactive sodium chloride was first called to our attention by Dr. William Duane, of Harvard University.

injected cannot be controlled exactly as an irregular quantity (25 to 50 per cent) will remain in the syringe. The rapid decay of the active deposit introduces a second source of error in estimating the quantity injected. Radium A decays completely within 15 minutes after the preparation of the solutions. Since our injections were made after 15 minutes, only Radium C need be taken into account. Radium C falls to 3 per cent of its initial value within 3 hours, so that the physiological activity of the injected solution can last only a relatively short time. The physiological effects are presumably due to the α -radiation.

EXPERIMENTAL.

Two animals were employed in the experiments described below. Dog 1 was a Dalmatian female of about 15 kilos. Benedict (2) has shown that this breed of dog regularly excretes large amounts of uric acid. We therefore used a Dalmatian in order to bring out any possible effect of the emanation upon the metabolism of this substance. Dog 2 was a bull-terrier female of about 12 kilos. Each animal was kept in a metal metabolism cage constructed for the proper separation and collection of urine and feces.

The diets employed for both dogs were made up of cracker meal, dog biscuit, evaporated milk, bone ash, and (in the case of Dog 2) a little casein. Dog 1 received 6 gm. of nitrogen and about 1,000 calories per day; Dog 2 received 5 gm. of nitrogen and about 660 calories per day.

The animals were catheterized daily just before feeding, and the bladder was washed out with distilled water followed by a little boric acid. The urine was collected under toluene and usually analyzed on the same day. The rectal temperature was taken and blood counts were made at frequent intervals. The injections were made into one of the superficial veins of the ear.

All analyses were made in duplicate; nitrogen was determined by the Kjeldahl-Gunning method, urea by a macro-urease method, ammonia by the older macro method of Folin. Creatinine was determined by the colorimeter method of Folin, uric acid by the Benedict-Hitchcock modification of the Folin-Denis method, and phosphates were determined by titration with uranium acetate. Albumin, sugar, and creatine were tested for frequently with negative results.

Experiment 1.

Table I records the experiments with the Dalmatian dog. The first 6 days (February 28 to March 5) serve as the control period before the first injection. On March 5 the dog received an intra-

venous injection of 95 millicuries of the active deposit. On the day of the injection there seemed to be no effects on the general condition of the animal but on the 2 following days the dog had to be coaxed to eat the food and the feces were softer than usual. After this no further general effects were present. An examination of the nitrogen partition shows a sharp rise in the total nitrogen output, which reaches its maximum on the 2nd day after the injection. This increased nitrogen is distributed among all the nitrogen constituents except creatinine. Ammonia increases proportionately more than does urea. Indeed the increase in the ammonia suggests a definite acidosis. The uric acid increases by about 50 per cent over that of the preliminary period. It is possible that this is associated with the destruction of white blood corpuscles which follows the injection. Phosphates and urinary volume both tend to show definite increases over the preliminary period.

The total nitrogen remains high for 5 days after the injection, and then drops suddenly. The ammonia and particularly the uric acid drop more slowly. The latter does not reach the level of the preliminary period until 12 days after the injection.

On April 4 the dog received a second intravenous injection of 30 millicuries of the active deposit. This dose is scarcely a third of the quantity given in the preceding experiment. The total nitrogen again increases, and again the maximal figure is reached on the 2nd day after the injection. The small dose employed in this experiment is followed by only a slight and transitory rise in uric acid. On the 3rd and 4th days after the injection creatinine shows an increase well above the preliminary period.

On April 15 the dog received its third injection of the active deposit. On this day 42 millicuries were given. This injection is followed by a marked and prolonged effect. Total nitrogen, urea, and ammonia increase and remain high for almost 2 weeks. Creatinine and uric acid both show a marked increase. The high figures for those constituents continue for about 8 days after the injection. Throughout the series of treatments on Dog 1, we find a steady increase in the volume of urine eliminated. At first the amount was 200 cc. and after the treatments it gradually rose to 1 liter.

TABLE I.

Period.	Date.	Weight of dog in gm.	Urine.												Titrateable 0.1 N acid.	Temperature. °F.	Remarks.
			Vol- ume.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Creatinine N.		Uric acid N.		P ₂ O ₅			
						gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent				
Con- trol.	1919	kg.	cc.														
	Feb. 28		210	1.028	4.21	3.58	85.00	0.241	5.73	0.094	2.23	0.101	2.40				Red cells 5,000,000 White " 11,640 Hemoglobin 85 % Red cells 4,400,000 White " 10,250 Hemoglobin 82% Red cells 4,120,000 White " 8,400 Red " 4,256,000 White " 7,450 Hemoglobin 70%
	Mar. 1		242	1.027	4.89	3.98	81.49	0.261	5.34	0.093	1.90	0.101	2.08				
	" 2-3		576	1.021	4.60	3.85	83.78	0.241	5.24	0.089	1.93	0.093	2.02	0.737			
" 4		480	1.016	4.83	4.13	85.60	0.219	4.53	0.096	1.98	0.094	1.95	1.05				
I*	"	5	210	1.030	4.55	3.84	84.30	0.250	5.49	0.098	2.15	0.101	2.23	1.00			
	"	6	390	1.025	5.26	4.51	85.84	0.236	4.49	0.096	1.82	0.130	2.48	1.22			
	"	7 14.77	590	1.016	5.43	4.56	83.91	0.351	6.51	0.097	1.79	0.155	2.85	1.11			
	"	8	510	1.018	5.21	4.41	84.74	0.331	6.36	0.098	1.84	0.135	2.60	0.902			
	"	9	390	1.019	5.14	4.44	86.40	0.304	5.91	0.094	1.83	0.159	3.10	0.853			Red cells 4,600,000 White " 4,800 Red " 4,640,000 White " 6,900
	"	10	348	1.022	5.13	4.43	86.60	0.255	4.99	0.094	1.84	0.159	3.11	0.728			
	"	11 14.88	327	1.020	4.51	3.83	85.00	0.281	6.23	0.094	2.09	0.125	2.77	0.894			
	"	12 15.00	395	1.019	4.65	3.82	82.15	0.253	5.44	0.098	2.10	0.113	2.48	0.759			
	"	13 14.88	490	1.018	4.73	4.02	84.90	0.222	4.69	0.101	2.14	0.122	2.57	0.780			
	"	14 14.77	392	1.020	4.92	4.07	82.57	0.269	5.47	0.099	1.99	0.126	2.56	0.863			
	"	15	438	1.017	4.55	3.85	84.69	0.247	5.43	0.095	2.10	0.113	2.49	0.749			

II†	"	215.11	345	1.021	4.88	4.1785.760.218	4.490.100	2.060.102	2.100.811	76	
	"	3	410	1.016	4.66	4.0286.500.235	5.060.088	2.110.095	2.050.852	104	
	"	4	395	1.016	4.86	4.1685.750.216	4.450.101	2.080.111	2.300.811	80	101.2
	"	5	508	1.015	4.89	4.2386.680.202	4.130.099	2.030.107	2.200.873	84	
	"	6	430	1.019	5.04	4.1983.200.261	5.180.118	2.340.101	2.000.998	92	99.8
	"	7	325	1.018	4.77	3.9983.530.196	4.110.121	2.540.101	2.130.831	76	
											Red cells 4,136,000 White " 10,200 Hemoglobin 80%
	"	8	518	1.014	4.66	4.0286.300.215	4.620.103	2.220.095	2.040.915	96	99.4
	"	9	15.30	1.016	5.18	4.3383.640.215	4.160.120	2.310.100	1.950.831	52	99.3
	"	10	500	1.014	4.72	3.8882.340.252	5.350.122	2.600.099	2.100.894	100	
III‡	"	11	15.30	1.014	4.97	4.1383.080.252	5.080.125	2.520.106	2.130.831	80	100.4
	"	12	382	1.016	4.63	3.9384.900.235	5.090.124	2.680.113	2.440.790	80	
	"	13-14	15.5	670	1.020	4.0986.200.202	4.260.124	2.610.109	2.310.831	80	100.6
											Red cells 4,680,000 White " 7,600 Hemoglobin 80%
	"	15	465	1.015	4.52	3.7582.860.241	5.340.126	2.800.113	2.510.749	88	
	"	16	550	1.017	5.24	4.4083.990.207	3.960.152	2.900.164	3.140.852	72	101.0
											Red cells 4,880,000 White " 6,150 Hemoglobin 80%
	"	17	15.2		5.46	4.6484.610.311	5.540.169	3.080.174	3.171.020	96	100.3
	"	18	15.4	535	1.015	5.17	4.3083.140.280	5.430.180	3.100.172	80	101.4
											100.7

* Injected 95 millicuries of active deposit at 1.00 p.m.

† " 30 " " 2.40 "

‡ " 42 " " 6.30 "

TABLE I—Concluded.

Period.	Date.	Weight of	Urine.												Titratable 0.1 N acid.	Temper- ature.	Remarks.
			Vol- ume.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Creatinine N.		Uric acid N.		P ₂ O ₅			
						gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent				
	1919	kg.	cc.												cc.	°F.	
	Apr. 19	15.2	678	1.012	5.05	4.24	83.89	0.252	5.00	0.159	2.97	0.161	3.19	0.811	60	100.8	
	" 20		570	1.010	4.91	4.13	84.13	0.275	5.60	0.150	3.06	0.151	3.08	0.811	60	101.1	
	" 21	15.4	750	1.012	5.40	4.63	85.67	0.255	4.72	0.155	2.87	0.129	2.39	0.790	64	100.7	
	" 22		640	1.011	4.85	4.17	85.93	0.287	5.92	0.151	3.04	0.133	2.76	0.790	64	100.7	
	" 23	14.7	670	1.013	5.39	4.58	84.90	0.224	4.16	0.144	2.68	0.124	2.30	0.852	64		
	" 28		485	1.017	5.42	4.55	83.99	0.264	4.87	0.120	2.22	0.104	1.93	0.790	96	100.6	
	" 29	15.3	600	1.011	5.19	4.48	86.18	0.255	4.92	0.103	1.99	0.104	2.00	0.811	60		
	" 30		848	1.009	5.08	4.39	86.36	0.230	4.53	0.101	1.99	0.111	2.19	0.706	64		
	May 1	15.3	848	1.011	5.42	4.66	85.93	0.233	4.29	0.108	2.00	0.112	2.08	0.786	75	99.3	
	" 2		830	1.010	5.67	4.87	86.95	0.207	3.66	0.109	1.93	0.106	1.87	0.748	72	100.4	
	" 3		850	1.008	4.98	4.26	85.50	0.238	4.90	0.112	2.32	0.127	2.55	0.764	67	100.0	
	" 7	15.5	1,004	1.009	4.91	4.22	85.87	0.198	4.04	0.117	2.39	0.125	2.55	0.786	73	100.5	
	" 8		1,005	1.010	4.86	4.23	86.98	0.188	3.87	0.114	2.36	0.127	2.61	0.786	63	100.4	
IV§																102.0	

§ Injected 64.5 millicuries of active deposit at 1.19 p.m.

On May 8 the animal received a fourth injection of 64 millicuries. This was followed by vomiting and refusal of most of the food for several days. The fact that the third and fourth injections were smaller in quantity than the first, and were followed by marked general effects, shows that the animal failed to recover completely from previous treatments with the emanation. Shortly after the fourth injection the animal was killed and an autopsy made. The autopsy findings will be reported in detail elsewhere.

Experiment 2.

Table II gives the results of injections of active deposit on the bull terrier; uric acid is eliminated only in very minute quantities, hence quantitative determinations of this constituent have been omitted. After a control period of 5 days, Dog 2 received the first injection of 120 millicuries. Urine voided voluntarily 15 minutes after the injection contained 9 millicuries of radioactivity. A decided rise in total nitrogen was noted which reached the maximum figure on the 2nd day. Urea rose proportionately with the total nitrogen and ammonia was decidedly increased. Total phosphates were almost doubled. White cells dropped from about 14,000 to 2,000 after the injection. The bull-terrier did not seem to bear the injections so well as the preceding animal for on the 3rd day following this treatment there was vomiting, diarrhea, and refusal of food. Determinations were omitted during this period. This dog also tended to lose weight throughout the experiment. 1 kilo was lost during this period so that creatinine which was high in the control period was considerably lower when the second injection of 17 millicuries was given.

Probably because of the decided effect produced by the first treatment 3 weeks previously, this small dose caused an appreciable rise in total nitrogen with a proportional increase in urea, ammonia, and creatinine, which was evident for 3 days. Total phosphates were somewhat increased.

10 days after the second treatment 54 millicuries were injected. This dose produced no general effect except that the animal refused part of the food on the 4th day. Total nitrogen, urea, and ammonia were considerably increased for 3 days and remained above normal for 10 days. Creatinine was not affected.

TABLE II.

Period.	Date.	Weight of dog.	Urine.										Titratable 0.1 N acid.	Temperature.	Remarks.	
			Vol- ume.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Creatinine N.		P ₂ O ₅				
			cc.		gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	cc.	° F.		
I*	1918															
	June 4	12.6	168	1.028	3.73	3.22	86.18	0.132	3.53	0.177	4.73	0.624	60	101.5	Red cells 6,400,000	
	" 5		158	1.032	3.86	3.31	85.86	0.115	2.98	0.171	4.43	0.624	44	101.0	White " 13,800 Hemoglobin 85%	
	" 6		288	1.018	3.78	3.28	86.52	0.179	4.74	0.177	4.67	0.707	64	101.0	Red cells 5,924,000 White " 14,400 Hemoglobin 85%	
	" 7		270	1.021	3.90	3.42	87.62	0.202	4.14	0.181	4.64	0.851	84	100.8		
	" 8-9		610	1.019	3.60	3.08	85.47	0.165	4.61	0.177	4.91	0.852	81	101.7	Red cells 5,600,000 White " 8,900 Hemoglobin 85%	
II†	" 10	12.5	300	1.020	4.34	3.68	84.69	0.141	3.23	0.181	4.17	0.790	60	101.3	Red cells 5,280,000 White " 4,550 Hemoglobin 80%	
	" 11		280	1.025	4.52	3.79	83.85	0.224	4.96	0.175	3.87	1.31	96	100.6		
	" 24		250	1.018	3.96	3.39	85.67	0.146	3.68	0.150	3.80	0.540	52	99.7		
	" 25		180	1.022	3.76	3.24	86.18	0.157	4.17	0.158	4.21	0.416	40			
	" 26		188	1.023	4.01	3.43	86.66	0.134	3.35	0.167	4.16	0.416	44	100.6		
	" 27		250	1.020	4.21	3.70	87.77	0.137	3.26	0.163	3.88	0.457	44	100.1		
	" 28	11.4	382	1.015	4.10	3.48	85.00	0.151	3.68	0.163	3.90	0.436	32	100.4		
	" 29-30		538	1.020	4.04	3.44	85.22	0.166	4.37	0.161	4.00	0.540	44	100.6	Red cells 4,976,000 White " 5,700 Hemoglobin 85%	
	July 1		243	1.022	4.56	3.86	84.55	0.213	4.67	0.179	3.92	0.623	72	100.4	Red cells 5,250,000 White " 4,450	

III†	"	4	11.9	140	1.030	4.10	3.62	88.45	0.165	4.04	0.156	3.82	0.540	48
	"	5		150	1.025	4.14	3.64	88.50	0.174	4.20	0.158	3.82	0.499	64
	"	6-7		400	1.022	4.11	3.60	87.62	0.148	3.61	0.149	3.62	0.561	52
	"	8		220	1.020	4.13	3.64	88.17	0.151	3.67	0.152	3.68	0.540	56
	"	9	11.9	155	1.028	4.14	3.63	87.62	0.162	3.93	0.158	3.82	0.519	56
	"	10		360	1.020	4.83	4.27	88.37	0.154	3.19	0.163	3.38	0.665	56
	"	11		390	1.017	4.80	4.18	87.08	0.199	4.15	0.158	3.29	0.665	68
	"	12	11.4	220	1.026	4.87	4.20	86.22	0.235	4.84	0.158	3.25	0.644	60
	"	13		205	1.025	4.43	3.92	88.43	0.148	3.35	0.162	3.64	0.561	52
IV§	"	14		295	1.017	4.15	3.54	85.21	0.207	5.00	0.163	3.93	0.520	52
	"	15		325	1.017	4.50	3.81	84.54	0.223	5.17	0.162	3.59	0.561	52
	"	16		385	1.016	4.43	3.77	84.90	0.219	4.94	0.156	3.53	0.541	60
	"	17	11.6	300	1.018	4.48	3.90	87.10	0.231	5.16	0.163	3.64	0.492	45
	"	18		335	1.017	4.55	3.95	86.78	0.193	4.26	0.153	3.37	0.478	56
	"	19		328	1.017	4.66	3.97	85.16	0.185	3.98	0.155	3.32	0.499	52
	"	20-		550	1.018	4.13	3.52	85.37	0.168	4.08	0.152	3.68	0.478	40
	"	21												
	"	22		415	1.014	4.24	3.57	84.28	0.190	4.50	0.153	3.62	0.519	64
	"	23	11.4	348	1.017	4.42	3.76	85.10	0.224	5.08	0.152	3.43	0.561	68
	"	24		410	1.013	4.17	3.67	88.03	0.176	4.24	0.158	3.79	0.478	40
	"	25		575	1.017	4.88	4.19	85.89	0.146	2.98	0.139	2.85	1.08	60

Red cells 5,800,000
White " 3,650
Hemoglobin 85%

Red cells 5,814,000
White " 4,100
Hemoglobin 80%

Red cells 5,200,000
White " 2,600
Hemoglobin 85%

* Injected 120 millicuries of active deposit at 7.15 p.m. on June 9.
† " 17.3 " " " 2.45 " " 30.
‡ " 54.7 " " " 4.45 "
§ " 148.4 " " " 3.00 "
|| No food eaten after this date.

2 weeks later, the last injection of 146 millicuries was given. All food was refused for 4 days, and when on the 5th day more food was eaten severe diarrhea immediately followed. The dog lost 1.5 kilos during this time. She was then anesthetized and an autopsy made.

DISCUSSION AND SUMMARY.

Our results record the effect of a purely physical agent, radium emanation, upon the metabolism. The experiment with Dog 1 is the most satisfactory, since in this animal the dosage employed was not great enough to produce general indisposition or sickness.

In every instance in both experiments the injection of active deposit was followed by an increased output of nitrogen, reaching the maximum figure on the 2nd day after the treatment. Urea fluctuates with the total nitrogen, but the absolute as well as relative amount of the total nitrogen excreted as ammonia nitrogen is decidedly increased, especially on the 2nd day after the treatment.

Much interest may be attached to the marked increase in creatinine which is noted in Dog 1. The quantity of this constituent is usually independent of volume and of total nitrogen. Shaffer (3) found some time ago that increased temperature had a tendency to increase creatinine but that it afterward went below normal. Van Hoogenhuyze and Verploegh (4) found an increase of 50 per cent in creatinine with no rise in total nitrogen in cases with a temperature. Leathes (5) found, on the other hand, that creatinine increased 20 per cent, but the total nitrogen increased 50 per cent in fevers. He concluded that the proportion of nitrogen excreted during fever appearing as uric acid was considerably increased but that as creatinine was consistently diminished. In our case the creatinine elimination does not fluctuate with temperature change—in fact the first increase in creatinine is noted after the second injection when the temperature has returned to normal. Uric acid at this time showed no increase. Although the highest creatinine values are noted on the days after the treatment when the nitrogen also is high, they do not increase proportionally to the total nitrogen. On the 2nd day of Period II nitrogen increased 3.5 per cent and creatinine 14 per

cent, and in Period III nitrogen increased 17 per cent while creatinine increased 28 per cent. On this day creatinine was 45 per cent higher than in the control period at the beginning of the experiment. The proportion of nitrogen as creatinine nitrogen was somewhat increased, but not greatly, after the treatment.

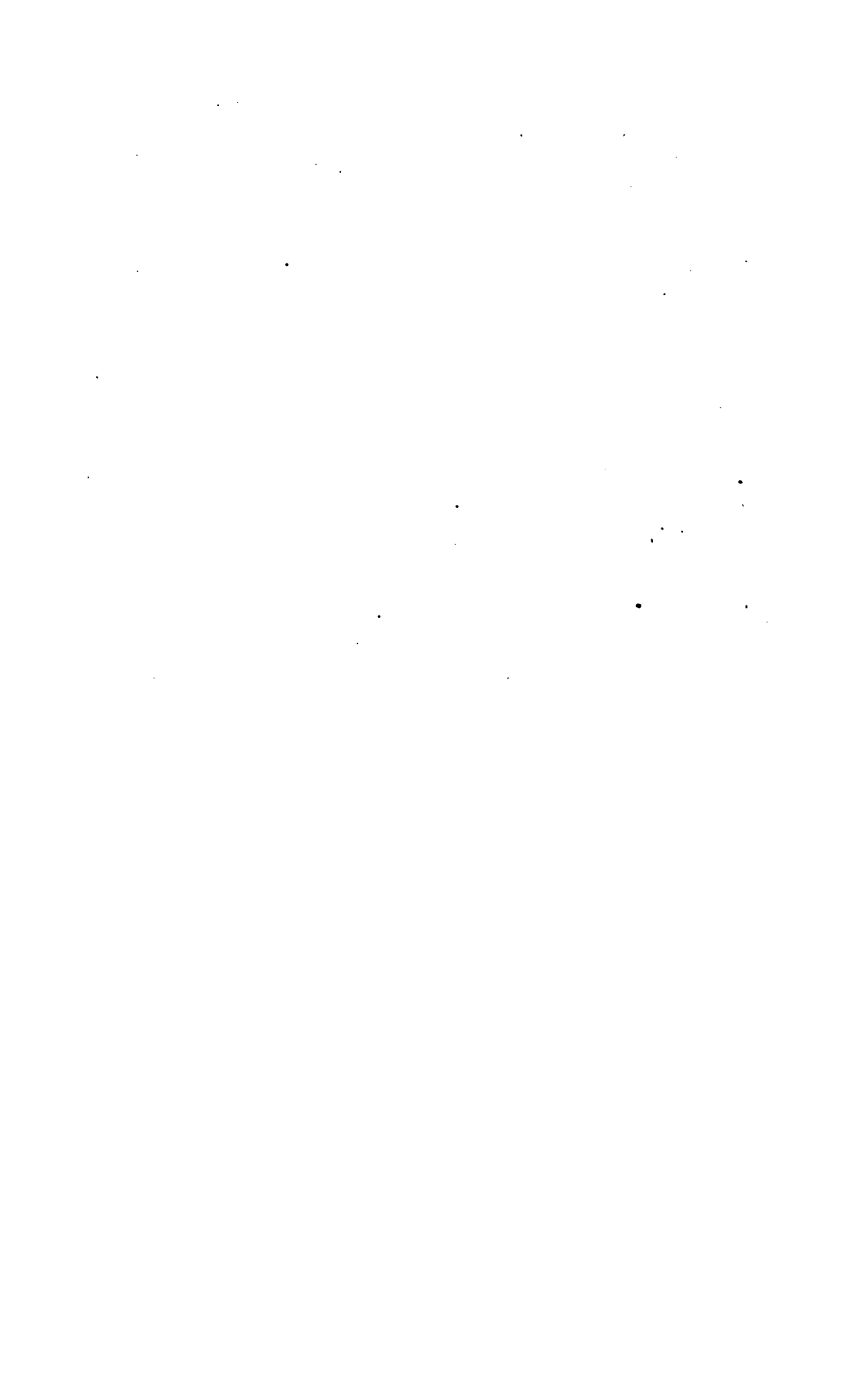
Uric acid showed a marked increase both absolutely and relatively following injection of radioactive deposit.

The effect of the injection seems to be cumulative because the third treatment in Dog 1, only a little larger than the second injection, produced a very marked effect, while the fourth treatment, although smaller than the first, produced nausea.

The writers wish to express their appreciation to the Radium Department for supplying the radium emanation and to Dr. Stanley R. Benedict for his suggestions during the work.

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THE DETERMINATION OF THE REFRACTIVITY OF HEMOGLOBIN IN SOLUTION.

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INTRODUCTION.

The refractivity of proteins in solution has been extensively investigated by Reiss, Herlitzka, Schmidt, and by Robertson and his collaborators. An excellent summary and discussion of these results, together with a bibliography, have been published by Robertson.¹ The following summary statement of facts recapitulates the main aspects of this work as far as they bear on the present problem.

(a) Proteins dissolved in water increase the refractive index of the solvent by an amount nearly proportional to the concentration of the protein. This approximately linear relation is expressed by the formula² $n - n_1 = a \times c$, where n represents the refractive index of the solution, n_1 the refractive index of the solvent, a a constant characteristic of the protein, and c the concentration of the protein. To determine the value of a for a given protein the refractive index of an aqueous solution of that protein in known concentration is first determined; from this value is subtracted the refractive index of the solvent, and the remainder is divided by the concentration; the quotient resulting is the value of a . If the value of a is known the percentage of that protein in a solution of unknown concentration can be calculated from the same formula.

¹ Robertson, T. B., *The physical chemistry of the proteins*, New York, 1918, 359.

² This formula does not express with entire precision the relation between n and c . It is based on the law of Gladstone and Dale which itself is only an approximation to experimental facts. It does, however, give the relation between n and c with a degree of inaccuracy not greater than the inherent error of refractometry under the conditions of this investigation.

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(b) It has also been shown that the increase in refractive index produced by a protein is not sensibly affected by the presence of other substances in solution provided no considerable molecular change in the protein, or of its degree of hydration or dispersion, is produced. Therefore the concentration of a protein in solution with other proteins, or with acids, bases, or salts in low concentration, can be determined by observing the index of refraction of the solution with and without the protein in question.

(c) The value of a for a considerable number of proteins has been determined. These values range from 0.0023 to 0.0013.

(d) There are certain limitations to accuracy in refractometric readings. The Pulfrich refractometer has an inherent error of 0.00009, while the error of the Abbe instrument is about the same, being approximately 0.0001. It is obvious therefore, as Robertson remarks, that, since the absolute error in the determination of $n - n_1$ is the same, the error in the determination of a must be less in proportion to the magnitude of c . It is therefore desirable to use as concentrated solutions as possible when determining the value of a . But maximum concentrations of proteins cannot be used because when a certain concentration is exceeded the border line seen in the field of the instrument becomes hazy and accurate setting of the instrument is impossible.

The present investigation was undertaken to find out whether the concentration of hemoglobin in solution might be estimated by the refractometric method, and to establish the value of a for hemoglobin. If this could be done it was obvious that a useful method might be devised for estimating hemoglobin in blood.

Methods.

The index of refraction was measured by an Abbe refractometer by Zeiss loaned by the Physics Department of Williams College through the courtesy of Prof. W. E. McElfresh. This type of refractometer, while less commonly used than is that of Pulfrich, is nevertheless very satisfactory. If allowed to attain the temperature of the room in which it is used, and if the room temperature is kept approximately constant, there is no difficulty in making several successive observations at the same temperature. The prisms are, however, water-jacketed and can be connected with a constant temperature reservoir if desired. This was not found necessary in the present work. All readings were made at 19–21°C., and in determining the value of $n - n_1$ the two readings necessary were made in quick succession and therefore at the same temperature. As the apparatus is provided with a compensating device to correct dispersion, a sodium flame or other source of monochromatic light is unnecessary. In this work an ordinary incandescent lamp was used as the source of light. The scale of the instrument is so constructed that the index of refraction

can be read directly from the graduated arc. The smallest subdivisions of the scale represent intervals of 0.001, but with some practice it is possible to estimate tenths of a subdivision with fair accuracy. It has therefore been assumed in this work that the error in reading is ± 0.0001 , but with care it is probable that the error can be reduced below this value. Several settings and readings were made for each determination and the average was taken. Consequently figures extending to the fifth decimal have occasionally been used with some confidence.

The work undertaken can be conveniently described in four parts; *viz.*, (A) preparation of pure hemoglobin, (B) examination of the validity of the formula $n - n_1 = a \times c$ when applied to hemoglobin solutions, (C) investigation of the possible effect of dilute alkalis and of serum constituents on the refractivity of hemoglobin, and (D) determination of the value of a for hemoglobin.

(A) *Methods of Preparation of Hemoglobin.*

Most of the crystallized hemoglobin used in this investigation was made by the method of Hoppe-Seyler as modified by Hüfner,³ and especially as modified in some details by Butterfield.⁴ In the case of hemoglobin from the guinea pig and the rat crystallization from laked blood took place readily in the cold without the addition of alcohol. Preparations from these species were, however, of little value for the purpose in hand because of their very sparing solubility. In the case of dog, horse, and beef bloods, alcohol was added to the laked blood, after cooling to 0°C., to make a concentration of from 20 to 30 per cent. The actual effective concentration was uncertain, and may have been higher than 30 per cent in some cases as ice crystals usually separated out at the low temperatures employed (-10 to $-20^\circ\text{C}.$) thus raising the concentration of alcohol in the solution. Crystallized hemoglobin was separated by centrifugalization, washed twice with 25 per cent alcohol, and finally dried on a porous plate *in vacuo* over H_2SO_4 at or close to 0°C.

Hemoglobin prepared in this way always contained a small and variable proportion of material insoluble in water but soluble in dilute alkali. This probably consisted of serum globulin and of stromata of corpuscles that had escaped solution by the ether, and that had been carried down by hemoglobin crystals in the centrifuge. It was found that after apparently thorough hemolysis of corpuscles by ether it was still possible to demonstrate stromata in the apparently clear liquid. If a drop of this liquid was mixed with a drop of saturated ammonium sulfate solution and examined under the microscope it was always possible to find some stro-

³ Hüfner, G., *Arch. Physiol.*, 1894, 134.

⁴ Butterfield, E. E., *Z. physiol. Chem.*, 1909, lxii, 173.

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mata, often in the typical bell-shaped form of erythrocytes. The salt did, of course, precipitate some of the hemoglobin, but there was no difficulty in distinguishing the stromata from the granular hemoglobin precipitate.

An effort was made to remove the stromata by filtration, but without success. Shaking the laked blood with filter paper pulp or with siliceous earth followed by filtration through asbestos felt at four atmospheres pressure was ineffective. As long as the solution came through the filter at all it carried stromata with it, but the flow stopped after a few cc. had filtered through. Centrifugalization was wholly ineffective in separating the stromata, as other investigators also have remarked.

When, however, hemoglobin prepared as above described was dissolved in water it was possible to separate the insoluble impurity by centrifugalization, to wash, dry, and weigh it, and then to calculate its percentage in the hemoglobin. But such a procedure probably gives inaccurate results and cannot be adopted with satisfaction.

After numerous unsuccessful efforts to get entirely consistent results with hemoglobin made as described, the paper of Welker and Williamson⁵ appeared calling attention to the method of removing colloids from solution devised by Marshall and Welker.⁶ The latter authors had shown that it is possible to remove entirely colloids *other than hemoglobin* from solutions by treatment with aluminum hydroxide. With this method, it was found that by shaking a laked corpuscle preparation with a freshly prepared mass of aluminum hydroxide, the colloids, other than hemoglobin, were adsorbed and suspended matter was entangled by the hydroxide and that a perfectly clear solution of hemoglobin, together with salts, could be filtered off with the utmost readiness. This method is of such excellence as to warrant the opinion that much of the work on hemoglobin in the past, in which discordant results have been obtained, might well be repeated using the method of Marshall and Welker for the preparation of the pure substance. All the observations included in the results published in the present paper, except where otherwise stated, were made on hemoglobin prepared from solutions thus purified. The technique of preparation was essentially that of Butterfield with the addition of the aluminum hydroxide purification after laking the blood and before the addition of alcohol.

⁵ Welker, W. H., and Williamson, C. S., *J. Biol. Chem.*, 1920, xli, 75.

⁶ Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, xxxv, 820.

(B) *Examination of the Validity of the Formula $n - n_1 = a \times c$ for Hemoglobin.*

As has already been stated, the refractivity of most proteins in solution is a linear function of their concentration. When the optical behavior of hemoglobin in this respect was examined it was found that within the limit of the inherent experimental error, the refractivity was directly proportional to the concentration. Experiment 1 demonstrates this agreement with the law.

Experiment 1.—The material used for this experiment consisted of twice washed horse corpuscles, laked with ether, then freed from ether by aspirated air, and finally treated with aluminum hydroxide. It contained about 17 per cent of hemoglobin and a small amount, probably about 1 per cent, of salts. 1 cc. of this solution was placed in each of six tubes, and to each tube was added from a calibrated pipette of 3 mm. bore enough distilled water to make the dilution 90, 80, 70, 60, 40, and 20 per cent respectively in the several tubes. After the liquid in each tube had been mixed, the refractive index was measured as well as the refractive index of the original solution. Table I gives the results of this experiment.

TABLE I.

	$n - n_1$	$\frac{n - n_1}{\text{relative concentration}}$
Original solution.....	0.0310	0.0031 ± 0.00001
90 per cent dilution.....	0.0282	0.0031 ± 0.00001
80 " " "	0.0251	0.0031 ± 0.00001
70 " " "	0.0216	0.0031 ± 0.00001
60 " " "	0.0187	0.0031 ± 0.00002
40 " " "	0.0124	0.0031 ± 0.00003
20 " " "	0.0062	0.0031 ± 0.00005

(C) *Refractivity of Hemoglobin in Water, in Dilute Alkali, and in Serum.*

As it was desired to use as solvents both dilute alkali and serum, it was necessary to find out whether the refractivity of hemoglobin was the same in each of the solvents. It had been shown by Gladstone and Dale⁷ that in general the refractivity of

⁷Gladstone, J. H., and Dale, T. P., *Phil. Trans. Roy. Soc.*, 1858, cxlviii, 8; 1863, cliii, 316.

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a solute is uninfluenced by other solutes provided no molecular change is induced, and Robertson⁸ has shown that this general truth applies to protein. The latter has further shown that the relatively slight alteration of molecular structure that occurs when proteins unite with acids or bases does not sensibly alter the refractivity, but, on the other hand, that the change in the degree of hydration that occurs when amorphous serum albumin solution is half saturated with ammonium sulfate does cause a notable change in the refractive index. With these facts in mind there would seem to be no reason to anticipate that the refractivity of hemoglobin in dilute alkali or in serum would be different from that in water, but the result of the experiment is conclusive. The refractivity of hemoglobin in these solvents is normal.

Experiment 2.—The refractive index of a solution of hemoglobin of about 17 per cent strength, and that of a 0.1 N NH_4OH solution were first determined, 1 cc. of each of these solutions was mixed together, and the refractive index of the mixture determined. It is evident that if the refractivity of the hemoglobin remained normal in the presence of 0.05 N NH_4OH then the refractive index of the mixture would be equal to the mean of the refractive indices of the component solutions. A parallel experiment with hemoglobin and serum was also made. The results follow.

Hemoglobin in Dilute Alkali.

(1) <i>n</i> of hemoglobin solution.....	1.3661
(2) <i>n</i> of 0.1 N NH_4OH	1.3341
Mean of refractive indices of (1) and (2).....	1.3501
<i>n</i> of mixture of equal parts of (1) and (2).....	1.3501

Hemoglobin in Serum.

(1) <i>n</i> of hemoglobin solution.....	1.3667
(2) <i>n</i> of serum.....	1.3440
Mean of refractive indices of (1) and (2).....	1.35535
<i>n</i> of mixture of equal parts of (1) and (2).....	1.3553

(D) Determination of the Value of α for Hemoglobin.

1. By Means of Solutions of Pure Crystallized Hemoglobin.—

Experiment 3.—Tubes 8 by 120 mm. each containing a glass bead were prepared and weighed. Crystallized dry hemoglobin was weighed in the

⁸ Robertson, T. B., *J. Biol. Chem.*, 1912, xi, 179.

tubes and the tubes were nearly filled with 0.1 N NH_4OH . The amount of hemoglobin was chosen to make approximately a 10 per cent solution. Solution was effected by warming in a bath at 30°C . and by thorough shaking. The tube was then centrifugalized to destroy the foam and a few drops of the solvent were added to facilitate observation of the meniscus. The level of the meniscus was carefully marked on the tube, the con-

TABLE II.

Concentration of Hb.	$n - n_1$	a
9.66	0.0178 ± 0.0001	0.001842 ± 0.00001
10.89	0.0198 ± 0.0001	0.001819 ± 0.00001
Average		0.001830 ± 0.00001

TABLE III.

Source of blood.	Concentration.	$n - n_1$
Guinea pig.	2.08	0.00365
	2.45	0.00425
Rat.	2.00	0.00320
	4.30	0.00770
Dog.	3.63	0.00630
	3.75	0.00610
	3.93	0.00700
	4.30	0.00755
	9.30	0.01800
Horse.	6.19	0.01160
	7.66	0.01360
Ox.	2.77	0.00485
	2.95	0.00555
	6.26	0.01065
	7.06	0.01275
	7.89	0.01385

Average value of $a = 0.00178 \pm 0.00002$. In determining the value of a from a number of observations due allowance should be made for the greater accuracy of the values obtained from the more concentrated solutions; i.e., a weighted average should be calculated. Following Robertson's suggestion this has been done by dividing the sum of the values of $n - n_1$ by the sum of the values of c .

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tents were thoroughly mixed, and the refractive index was read. The tube was then washed and filled with distilled water to the mark, and weighed. The volume of the tube to the mark divided into the weight of hemoglobin gave the concentration of hemoglobin in the solution of which the refractive index had been read. From these data the value of a was calculated. Table II gives the results of two determinations on horse hemoglobin.

Before becoming acquainted with the aluminum hydroxide method of preparing pure hemoglobin, sixteen separate determinations of the value of a were made on specimens of hemoglobin prepared from the blood of the rat, guinea pig, dog, horse, and ox. It seems worth while to include the results of these observations (see Table III), although the value of a obtained is affected by the presence of the insoluble impurity to which reference has already been made, and is consequently slightly lower than the correct value given in Table II.

2. By Means of Hemoglobin Solutions Freed from Salts by Dialysis but Not Crystallized.—The hemoglobin molecule is very sensitive to denaturing influences. While the uniformity of results obtained by different recent observers who have determined the spectrophotometric constant of hemoglobin would incline one to the conclusion that they were dealing with a sufficiently stable substance, yet the divergence of results of proximate analyses make it possible that either the alcohol used to bring about crystallization, or the subsequent drying, results in a preparation that is to some extent dehydrated or denatured, and possibly with an altered refractivity. With this possibility in view the value of a was calculated from hemoglobin that had not been subjected to any dehydrating agent and that had not been crystallized. The following procedure was employed.

Experiment 4.—Ether-laked blood was purified by aluminum hydrate and then dialyzed in a Schleich and Schüll thimble until the dialysate gave no precipitate with AgNO_3 , and its refractive index was the same as that of pure water. Such a solution is free from colloids and suspended matter, and sufficiently free from salts for the purposes of this investigation. The value of $n - n_1$ (n_1 of distilled water) for this solution was determined and then an accurately measured volume was evaporated in a drying oven, and the weight of the dry residue determined in the usual way. The concentration of hemoglobin was calculated from the volume of the solution and the weight of dry residue. The following result was obtained.

Concentration of Hb.

per cent

 $n - n_1$ α

13.23

0.0242

 0.00183 ± 0.000008

3. *By Refractometric Comparison of Laked and Unlaked Blood, with Gasometric Determination of Hemoglobin.*—Unlaked blood offers an exceptionally favorable opportunity for measuring the refractivity of hemoglobin; for in unlaked blood the hemoglobin, remaining in the corpuscles, does not affect the refractive index, the refractive index of whole blood being identical with that of the plasma.⁹ On laking, the hemoglobin is discharged from the corpuscles, and entering into solution in the plasma raises its refractive index by an amount proportional to the percentage of the hemoglobin in the blood. This statement involves the assumption that freezing and thawing cause, first, the complete discharge of all the hemoglobin, and, second, an inappreciable discharge of electrolytes and water. The first assumption can be readily tested. It was found that alternately freezing and thawing blood three times caused a rise in the refractive index that was not increased by further freezing and thawing, and from blood so treated no sediment could be obtained on centrifugalization. The extent to which water and electrolytes are discharged from the corpuscles by hemolysis cannot be so easily determined. Stewart¹⁰ has observed the change in conductivity of serum following hemolysis produced by various agencies, and has concluded that freezing and thawing cause a less discharge of electrolytes than does any other agency. He was unable to estimate with precision the possible increase of electrolytes in the serum because the entrance of hemoglobin itself into the serum lowers the conductivity by opposing the migration of ions and by putting back the dissociation of electrolytes. From the results of Stewart's work, however, it seems probable that any change in the refractivity of the serum due to the entrance of water and electrolytes from the corpuscles must be of a very low order of magnitude. Moreover the concordance between the result of the present experiment, based on this assumption,

⁹ When whole blood is first observed in the refractometer the border line is very hazy, but within 30 seconds it becomes quite sharply defined.

¹⁰ Stewart, G. N., *J. Physiol.*, 1899, xxiv, 211.

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and the results of other methods of determining the value of a speaks for the correctness of the assumption itself.

The gasometric method of determining the oxygen of blood devised by Van Slyke¹¹ furnishes an accurate method of estimating the percentage of hemoglobin, for it is known that 1 cc. of oxygen combines with 0.744 gm. of hemoglobin.¹²

Experiment 5.—The oxygen capacity of human blood, oxalated and thoroughly oxygenated by rolling for 20 minutes in a large bottle, was measured in the Van Slyke apparatus. Only two drops of octyl alcohol, redistilled at 35 mm. pressure and tested, were used, and saponin was dispensed with to make it practicable to use so small an amount of foam preventer. The average of five closely concordant readings was 0.418 cc. The calculated amount of oxygen was 16.97 cc. per 100 cc. of blood, and the calculated percentage of hemoglobin 12.625 (16.97×0.744).

1 cc. of the same blood was sealed in a tube and alternately frozen and thawed four times. The refractive index of the perfectly clear liquid was then measured and found to be 1.3697. The refractive index of the whole blood (or of the plasma) was 1.3466. The difference between these two figures, the refractivity of the hemoglobin, is 0.0231, and this number divided by the concentration of the hemoglobin (12.625) gives 0.00183 ± 0.000008 , which is the value of a .

CONCLUSIONS.

The object of this investigation was the determination of the value of a for hemoglobin. When reached by three separate and distinct methods the result has proved to be the same; namely, $a = 0.00183$. It is therefore believed that this figure can safely

¹¹ Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347; 1918, xxxiii, 127.

¹² It has been conclusively shown by Peters (Peters, R. A., *J. Physiol.*, 1912, xlv, 131) and by Barcroft and Burn (Barcroft, J., and Burn, J. H., *J. Physiol.*, 1913, xlv, 493) that loosely combined oxygen and iron are associated in oxyhemoglobin in the proportion of 1 molecule of oxygen to 1 atom of iron; and Butterfield (Butterfield, E. E., *Z. physiol. Chem.*, 1909, lxii, 173) has determined the percentage of iron in oxyhemoglobin to be 0.335 per cent. Calculation from these data shows that 1 cc. of oxygen is combined with 0.744 gm. of hemoglobin.

No account has been taken in this work of the theoretical difference in the refractivities of oxyhemoglobin and hemoglobin. As molecular refractivity is an additive function of atomic refractivity the introduction of 2 atoms of oxygen into the enormous hemoglobin molecule would affect the refractivity to a quite inappreciable extent only.

be used to calculate the strength of hemoglobin solutions of unknown concentration when the value of $n - n_1$ can be found.

With the value of a determined, and with the knowledge that the difference in the refractive indices of laked and unlaked blood is due practically entirely to the hemoglobin, it is evident that a simple and fairly accurate clinical method of estimating hemoglobin in blood can be devised. The details of a clinical method, employing saponin hemolysis, are now being worked out. This technique together with data regarding the degree of accuracy obtainable will be communicated later.

SUMMARY.

1. It has been shown that the refractive index of solutions of hemoglobin varies directly with the concentration.
2. The refractive index of hemoglobin in aqueous solution has been shown to be independent of the presence of other proteins, and of bases and salts in low concentration.
3. The value of a for hemoglobin has been determined to be 0.00183.
4. The possibility of a practical clinical method for the refractometric estimation of hemoglobin has been indicated.

The advice and collaboration of Professor G. A. Shook, now of Wheaton College, Norton, Mass., but at Williams College at the beginning of this investigation in 1917, were of great value. On the resumption of the investigation, interrupted by the war, Professor Shook's absence made his cooperation no longer possible. He has, however, independently devised and reported¹³ a differential refractometer which it is believed can be conveniently used with this method.

The facilities of the Chemical Laboratory of Williams College were made available when necessary for this work through the kindness of Professor Brainerd Mears.

¹³ Shook, G. A., *J. Ind. and Eng. Chem.*, 1918, x, 553.

NUTRITIVE FACTORS IN PLANT TISSUES.*

IV. FAT-SOLUBLE VITAMINE.

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The occurrence of fat-soluble vitamine in green foods has been demonstrated for alfalfa, clover, and timothy plants and for the leaves of spinach and cabbage.¹ From some of these we have obtained potent products by drying the plant tissues in a current of air at about 60° and then extracting them with u. s. p. ether.² The resultant green extracts from spinach leaves, young clover, alfalfa, and grass respectively yielded an oily residue approximating 3 per cent of the dried plant.³ These residues, fed in daily quantities equivalent to 1 to 2 gm. of the dried plant, promoted recovery and renewal of growth in rats declining in weight on diets deficient in fat-soluble vitamine. McCollum, Simmonds, and Pitz⁴ have stated that "ether extraction of plant tissue does not remove the substances essential for growth which is contained in butter fat." The results of our successful experience,

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

¹ For a discussion of the literature see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187.

² Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1918-19, xvi, 98.

³ The actual content of substance removed from a number of samples of dried plant tissues in this way was as follows: spinach, 3.0 and 4.3 per cent; young alfalfa, 3.0 per cent; green grass, 4.1 per cent; timothy, 4.2 per cent; tomato, 2.8 per cent.

⁴ McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 363.

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however, are illustrated in Chart I. The food mixtures had the composition as given in Table I.

Although dried tomato was efficacious as a source of fat-soluble vitamine, its ether extract was not (see Chart II).

Recently Steenbock and Gross⁵ have concluded that:

" . . . tubers and roots are not necessarily to be classed with food materials grossly deficient in their fat-soluble vitamine content. While in some instances it is true that there is little or no fat-soluble vitamine demonstrable, in other instances there is enough present to warrant their classification with respect to their content of this dietary essential with leafy materials rather than with our cereal grains such as maize, wheat, barley, or oats."

TABLE I.

Food:	Rats 5411, 5412, 5417, 5425, 5430.	Rats 5570, 5563.	Rat 5527.
	per cent	per cent	per cent
Meat residue.....	19.6		
Casein.....		18.0	
Lactalbumin.....			18.0
Salt mixture*.....	4.0	4.0	4.0
Starch.....	52.4	48.0	47.0
Lard.....	24.0	30.0	31.0
Yeast.....	0.2-0.6 gm. daily.	0.4 gm. daily.	0.4 gm. daily.

* The composition of the salt mixture is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

We are not familiar with any published determinations of the *absolute* quantity of carriers of fat-soluble vitamine necessary to keep growing animals in health and vigor. It has been reported that for rats 2 per cent of butter fat is sufficient for the maintenance of good growth when all other dietary factors are of good quality,⁶ although Drummond⁷ who has had large experience in this field has failed to secure normal growth of rats with even 4 per cent of butter fat in an otherwise suitable diet. Early in our studies of the fat-soluble vitamine we⁸ secured good growth for

⁵ Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 529.

⁶ McCollum, E. V., Simmonds, N., and Parsons, H., *J. Biol. Chem.*, 1919, xxxvii, 162.

⁷ Drummond, J. C., *Biochem. J.*, 1919, xiii, 81, Experiments 1 to 10, 85.

⁸ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.

long periods on diets containing 1 and 3 per cent of butter fat respectively along with protein-free milk; ultimately a decline ensued and body weight was restored by increasing the content of butter fat in the food mixture.

Without knowledge as to the total food intake it is impossible to estimate the absolute amount of butter fat eaten with any approach to accuracy. However, rats usually eat enough of diets thus poor in fats to make it unlikely that the minimum quantity of butter fat ingested daily in the case of the 2 per cent fat ration would be less than 150 to 200 mg.

In contrast with this we have observed that only 42 mg. per day of the U. S. P. ether extract of grass sufficed to furnish enough fat-soluble vitamine to promote renewal of growth in rats that had declined on diets deficient in this factor. Furthermore in a number of instances the now familiar eye disease that frequently afflicts rats which have been for some time on a diet deficient in fat-soluble vitamine was cured after the administration of ether extracts of alfalfa, grass, or spinach (see Chart I, Rats 5417, 5570, 5425, 5563).

The foregoing observations have raised the question as to the comparative potency of green vegetables, roots, *etc.* as sources of fat-soluble vitamine in contrast with the more familiar carriers represented in the fats of milk, egg, *etc.*—products already demonstrated to furnish this essential food factor. To secure evidence we have fed rats, beginning with approximately the same age and size, on a diet complete in respect to each known essential except fat-soluble vitamine. The basal food mixture consisted of:

	<i>per cent</i>
Meat residue.....	19.6
Salt mixture*.....	4.0
Starch.....	52.4
Lard.....	24.0
Yeast.....	0.4 gm. daily.

* The composition of the salt mixture is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The quantity of brewery yeast added as a source of water-soluble vitamine has been demonstrated by us to be ample and

also practically free from fat-soluble vitamine.⁹ Upon the diet outlined rats soon fail to grow and then begin to decline rapidly in body weight, as shown in Chart III, Rats 5496, 5491, and 5512. Unless the physiological damage has proceeded too far, restoration of growth and well being is usually readily brought about by inclusion of butter fat in place of part of the lard in the food mixture. The characteristic eye conditions or symptoms, sometimes termed xerophthalmia or perhaps better keratomalacia, which often develop during the decline in health,¹⁰ are speedily relieved when the nutritive conditions begin to improve.

To test the comparative efficacy of various products as sources of fat-soluble vitamine they were fed, apart from the food mixture, in daily doses of approximately 0.1 gm. of the dried substance.¹¹ These were consumed with readiness by the animals so that a fairly constant supply of the product to be tested was always ingested, despite variations in the total food intake.

The products used were prepared from the plant materials by heating in a large drier, through which a current of air circulated at 60° or less, and grinding the dry residues to a powder.

The changes in body weight of the animals in this series of experiments are shown in Charts IV to XI. Whenever any of the animals began to decline in body weight and thus give evidence of unsatisfactory nutrition the diet was changed by the addition of 18 per cent of butter fat to the food mixture, in order to ascertain whether a shortage of the fat-soluble factor was the real cause for the failure of maintenance.

To permit comparison with a more familiar source of fat-soluble vitamine a series of rats was fed 0.1 gm. of butter fat daily instead of the dried vegetable. With a food intake of 50

⁹ We have referred to this fact in earlier papers; *e.g.*, Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 158; 1919, xxxvii, 199. See also Drummond, J. C., *Biochem. J.*, 1917, xi, 255.

¹⁰ We cannot accept the implication of Bulley, E. C., *Biochem. J.*, 1919, xiii, 103, that lack of fat-soluble vitamine plays no decisive rôle in the appearance of the symptoms of eye disease. Our evidence will be presented at another time.

¹¹ The materials were measured with small scoops which did not deliver exact quantities. The \pm variations were found subsequently to amount to 40 per cent in a few instances. This does not materially alter the significance of the results.

to 70 gm. per week, which we¹² have found usual for animals of the same size on adequate foods of similar calorie value, this amount of butter fat would be equivalent to that in a mixture containing 1.4 to 1.0 per cent thereof. Chart III contrasts the records of rats that received no butter fat and began to decline in 50 to 60 days with the weight curves of three rats which were fed 0.1 gm. of butter fat daily and reached a maximum weight of 290, 310, and 320 gm. respectively before giving any indication of qualitative inadequacy of their diet. Rat 5300 could no longer be restored by the administration of butter fat; the other two animals, however, showed a slight response to this product, indicating that they may have suffered somewhat from a lack of the fat-soluble factor found therein.

The growth of rats to adult size upon diets in which the daily intake of butter fat as a source of fat-soluble vitamine was as small as in the experiments just described was surprisingly good. It should be borne in mind that, in contrast with what is true of animals having declined through complete deprivation of some requisite food factor, the problem of dosage for vitamins may be quite different in the case of rats which are supplied with a minimum of the essentials throughout the period of growth. When the other dietary factors were satisfactory we have repeatedly found 0.5 gm. of butter fat per day to be sufficient to restore to good nutritive condition and growth rats declining on diets devoid of fat-soluble vitamine. This corresponds, for our food mixtures, to a butter fat content of about 5 per cent. How much less might suffice, we cannot state at present.

If butter fat, which has been studied more extensively than any other source of fat-soluble vitamine, is used as a standard for comparison the relatively large content of fat-soluble vitamine in some of the vegetable products examined is at once suggested by inspection of our records. For example, two rats (Nos. 5385 and 5386, Chart IV) receiving only 0.1 gm. of tomato daily have rapidly grown to exceptionally large adult size and have not shown any signs of a failure of nutrition at the end of 394 days. In considering this unexpectedly good physiological performance it must be remembered that the more rapid growth

¹² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

at the start may have been facilitated by the additional supply of water-soluble vitamin B (water-soluble B) furnished by the tomato, which we¹³ have demonstrated to be rich in this factor.

It may be necessary to take into consideration another factor supplied by the dried tomato. Givens and McClugage¹⁴ have shown that the latter, in contrast with some other vegetable foods, may retain a significant amount of its antiscorbutic potency. Is the exceptionally good growth of the tomato-fed rats attributable to this? Although it has generally been assumed that rats are not susceptible to scurvy both Harden and Zilva¹⁵ and Drummond¹⁶ maintain that this species also requires antiscorbutic vitamins. Thus Harden and Zilva conclude:

" . . . rats existing on a scorbutic diet, although capable of gaining in weight and reproducing themselves, without any apparent manifestation of pathological symptoms for months, do not thrive so well as animals which have their diets supplemented with an antiscorbutic. This suggests that although rats are not very susceptible to scurvy they cannot absolutely dispense with antiscorbutics without restriction of their normal development."

The evidence of all these English investigators consists in the demonstration of the attainment of larger size by rats which received orange juice or lemon juice as an antiscorbutic in addition to yeast as a source of water-soluble vitamin B. It is not clear, however, that the fruit products did not function merely as added sources of water-soluble B which we have found them to contain,¹⁷ and thus promote the rate of growth by the increment of a factor other than the antiscorbutic. We have frequently observed improved growth in rats when the supply of vitamin B from yeast was increased. Without a demonstration that more yeast, which is not regarded as antiscorbutic, or some other source of water-soluble B should fail to accomplish what is claimed for orange juice added to yeast, the conclusion of the

¹³ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187; 1920, xli, 451.

¹⁴ Givens, M. H., and McClugage, H. B., *J. Biol. Chem.*, 1919, xxxvii, 253.

¹⁵ Harden, A., and Zilva, S. S., *Biochem. J.*, 1918, xii, 408.

¹⁶ Drummond, J. C., *Biochem. J.*, 1919, xiii, 77.

¹⁷ Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 46.

need of some special antiscorbutic by rats is not convincing. We have on several occasions attempted without success by giving orange juice or tomato to promote the growth of rats that failed to respond properly to yeast as a source of water-soluble vitamine. However, it should be pointed out that occasionally animals which fail to grow on our food mixtures as well as one might expect begin to thrive when a mixed diet is furnished. The reason for this is not yet clear.

Among the various dried vegetables tested for their comparative content of fat-soluble vitamine the cabbage was least satisfactory (see Rats 5247, 5248, 5297, Chart V). The ready response of two of the animals to additions of butter fat to the diet indicates the character of the deficiency in the cabbage. 0.1 gm. of alfalfa, clover, timothy, and spinach evidently furnishes relatively at least as much of this vitamine as does 0.1 gm. of butter fat (see Charts VI, VII, VIII, and IX). These vegetable products may in fact contain more than butter fat.

Some of the animals receiving the dried green products grew to large adult size before evincing any signs of a possible shortage of fat-soluble vitamine. Eye disease characteristic of a lack of this vitamine was not observed in any of these animals.

Tests with dried carrots are shown in Chart X. The experiments with potato (including the skin), Chart XI, are not strictly comparable with the others inasmuch as the quantities of the tuber used, *i.e.* 20 per cent of the food, were large. The large size attained by two of the rats on the potato food indicates that the tuber as a whole cannot be entirely devoid of fat-soluble vitamine.

In an investigation of the distribution of fat-soluble vitamine in some roots Steenbock and Gross⁵ have recently concluded:

"With 15 per cent of the diet made up of roots as the source of the fat-soluble vitamine we have in the case of the yellow sweet potato and carrot normal growth and even rearing of the young made possible, but in the case of the rutabaga, dasheen, red beet, parsnip, potato, mangel, and sugar beet complete failure resulted."

In the experiments of these investigators larger quantities of potato in the diet, however, permitted growth, this corresponding with our observation that this tuber, though poor in fat-soluble vitamine, is by no means entirely devoid of it.

Early in our investigation of the properties of butter fat we reached the conclusion that the fat-soluble vitamine as it occurs in this natural product is not readily destroyed by heating with steam. "Butter fat through which live steam was passed for two and one-half hours or longer did not lose its characteristic restorative properties"¹⁸ when fed to rats which had declined on diets deficient in fat-soluble vitamine. We have since duplicated this observation.

Steenbock, Boutwell, and Kent¹⁹ and Drummond⁷ have reached the conclusion that the fat-soluble vitamine is readily destroyed by heat. Thus Drummond states, in confirmation of the claims of the American investigators:

"Exposure of butter fat to 100° for periods of from one to four hours destroys its growth promoting power entirely, so far as can be determined by experiments on young rats The effect of lower temperatures was also investigated and it was ascertained that the nutritive value of butter fat may be appreciably lowered by four hours' exposure to temperatures ranging from 50°-75°."²⁰

In comparing these statements with the findings which we published earlier it should be noted that the newer experiments have involved heating the fat *in the absence of water*. Our heated butter fat was subjected to steaming, not dry heat, for several hours. It seemed possible that the differences in the mode of heating, involving unlike possibilities of dehydration, *etc.*, might account for the discrepancies recorded. However, in experiments which will be reported in another paper we have since heated dry butter fat in an air bath at 96° for 15 hours without destroying sufficient fat-soluble vitamine (if any) to make the product appear inferior to the original butter fat when tested on rats that have declined from lack of fat-soluble vitamine.

We are not prepared to say that heat is without effect upon this vitamine, because we have not yet tested heated butter fat in quantities sufficiently small to meet the valid objection that despite some destruction sufficient amounts still remained in the

¹⁸ Osborne and Mendel, ⁸ p. 381.

¹⁹ Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.

²⁰ Drummond,⁷ p. 86.

large portion of heated fat fed to satisfy all requirements. We do know, however, that our animals ate all the heated butter fat apportioned to them, because it was fed apart from the food and not incorporated in the rest of the ration. In considering Drummond's experiments one is struck by the fact that even with 6 per cent of unheated butter fat in the diet²¹ his control rats grew at much less than the normal rate. This is contrary to the experience of several investigators, including ourselves, and raises a question as to the value of the untreated butter fat or the food intake of the animals used by him. At any rate the reason for the discrepancies between us are not apparent.

We have referred here in detail to some of these problems in connection with the effect of heat on the fat-soluble vitamine because it might be assumed that the low heat employed in desiccating the fresh foods studied in this research had diminished their content of fat-soluble vitamine. It is known that cabbage loses its antiscorbutic potency through drying; and this naturally suggests that the poor showing made by dried cabbage as a source of fat-soluble vitamine might also be ascribed to deterioration through heat and desiccation. The positive results secured with the other dried foods speak against such a conclusion. We have not yet made the crucial comparisons between the fresh and dried products. Drummond⁷ states in respect to his own observations that "the experiments with cabbage gave evidence that drying may reduce the efficiency of leaves as a source of fat-soluble A, but no definite opinion can yet be given." Drummond refers to the experiments on guinea pigs by Delf²² and Delf and Skelton²³ which lead them to believe that high temperature or the drying of cabbage leaves may effect a destruction of the fat-soluble accessory. Their experiments, however, seem to us to lack adequate controls and are not convincing. More recently Steenbock and Gross have asserted, in contrast with their findings regarding the thermolability of the vitamine in butter fat, that "the fat-soluble vitamine as found in plant materials was very stable to heat."²⁴

²¹ Drummond,⁷ p. 86, Experiment 6, Table I.

²² Delf, E. M., *Biochem. J.*, 1918, xii, 416.

²³ Delf, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 448.

²⁴ Steenbock and Gross,⁵ p. 506.

558 Nutritive Factors in Plant Tissues. IV

The newer studies indicating the richness of many types of plant tissues in those nutritive properties termed vitamins place the dietary importance of the green vegetables in an entirely new light. It emphasizes their use to supplement the refined foods of the modern food industry which furnish products rich in proteins, fats, and carbohydrates but in many cases comparatively deficient in the vitamins. The facts cited in the present investigation, along with others recently published, serve as an added reminder that the fat-soluble vitamin need not be sought solely in foods known to be rich in fats.

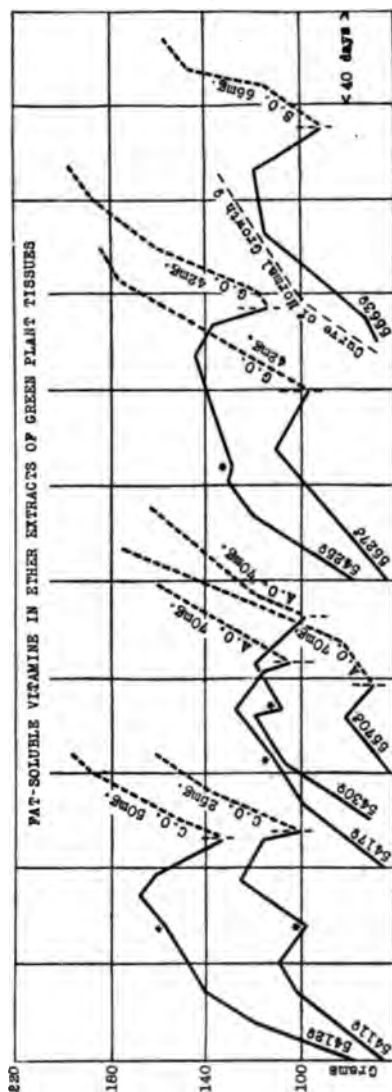


CHART I. Showing the efficiency of u. s. p. ether extracts from clover, alfalfa, grass, and spinach respectively as sources of fat-soluble vitamins in promoting restoration of growth in rats that had begun to decline on diets otherwise adequate. An asterisk (*) marks the period at which the daily allowance of dried brewery yeast, fed apart from the rest of the food, was increased to 0.6 gm. Note that this addendum alone sufficed to promote the growth of the animals for a time in some instances, although decline ultimately ensued in every case. Rats 5417, 5570, 5425, and 5563 developed the characteristic eye disease, which in each case disappeared after the administration of the plant oil. Interrupted lines indicate the periods during which the plant oils were fed mixed with starch and apart from the rest of the ration. C.O. = clover oil; A.O. = alfalfa oil; G.O. = grass oil; S.O. = spinach oil. The daily dose is indicated on the chart.

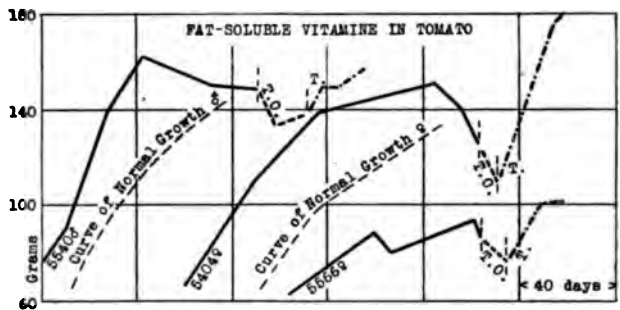


CHART II. Showing the failure of the ether extract of dried tomato (T. O.), in the quantities used, to promote renewal of growth in animals that had declined on a diet deficient in fat-soluble vitamines; and the renewal of growth when 1 gm. of dried tomato (T.) was furnished daily. The composition of the foods was as follows:

	Rat 5440♂.	Rat 5404♀.	Rat 5566♀.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein.....	18.0		18.0
Meat residue....		19.6	
Salt mixture*....	4.0	4.0	4.0
Starch.....	48.0	52.4	48.0
Lard.....	30.0	24.0	30.0
Yeast.....	0.4 gm. daily.	0.2-0.6 gm. daily.	0.4 gm. daily.
Tomato oil.....	56-112 mg. "	56 mg. "	112 mg. "

* The composition of the salt mixture is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

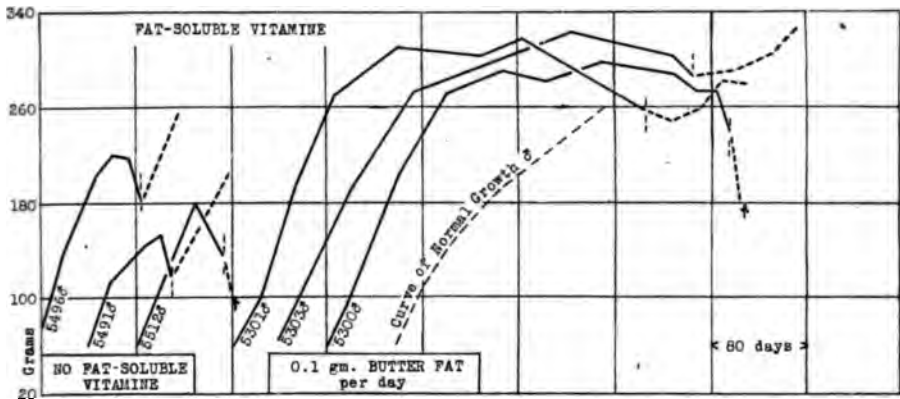


CHART III. Showing early failure to grow and decline of Rats 5496, 5491, and 5512 on a diet deficient in fat-soluble vitamin; also better growth of Rats 5301, 5303, and 5300 when 0.1 gm. of butter fat was supplied daily from the beginning of the experiment. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet.

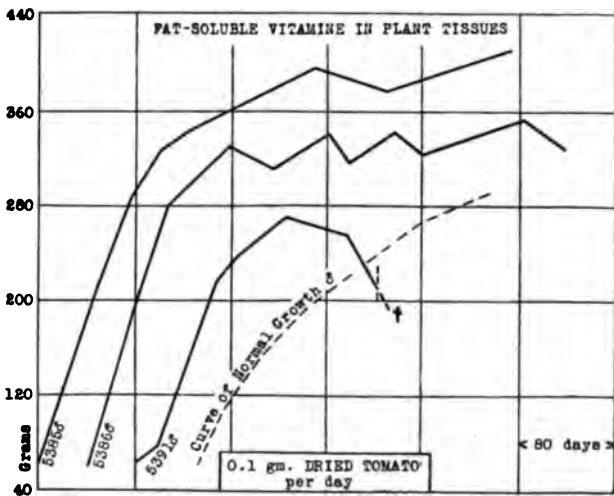


CHART IV. Showing the exceptional growth of rats on a diet containing 0.1 gm. of dried tomato as the source of fat-soluble vitamin. They represent the most successful growth observed in the entire series in this paper. Rat 5391 died of lung disease.

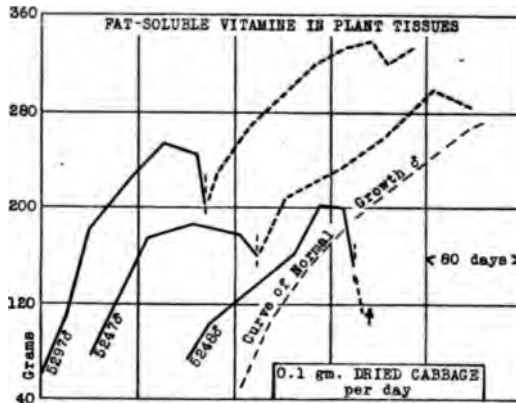


CHART V. Showing the inadequacy of 0.1 gm. of dried cabbage per day to supply sufficient fat-soluble vitamine to permit prolonged growth. The nature of the deficiency is shown by the renewal of growth when 18 per cent of butter fat was included in the diet during the period represented by the interrupted lines. That this amount of cabbage is not entirely lacking in fat-soluble vitamine is shown by the contrast of these results with the control experiments without fat-soluble vitamine, represented in Chart III.

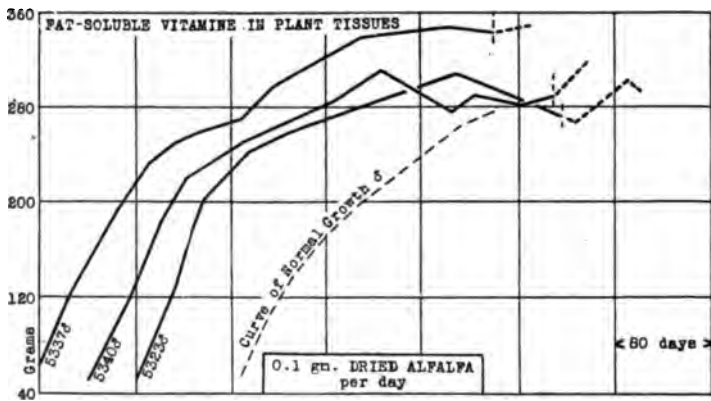


CHART VI. Experiments in which 0.1 gm. of dried alfalfa was fed daily in addition to a diet deficient in fat-soluble vitamine. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet.

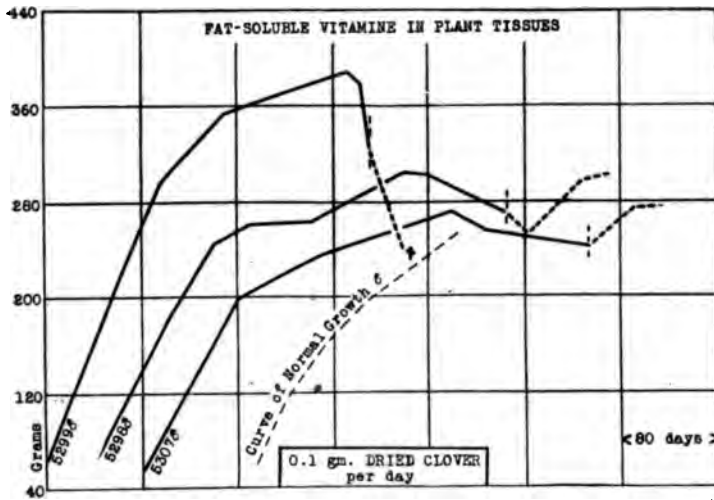


CHART VII. Experiments in which 0.1 gm. of dried clover was fed daily in addition to a diet deficient in fat-soluble vitamin. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet. Rat 5299 died of lung disease.

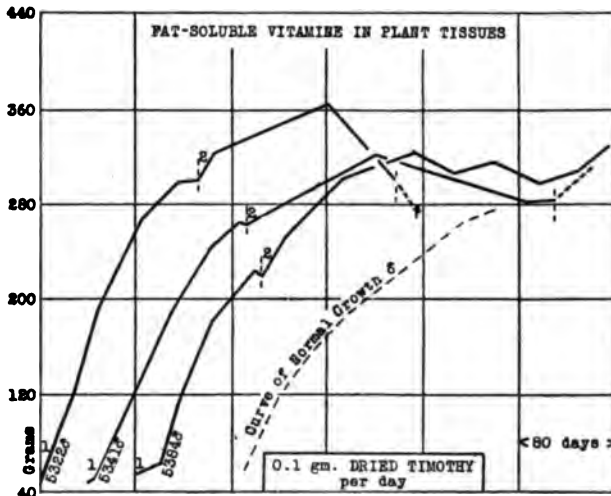


CHART VIII. Experiments in which 0.1 gm. of dried timothy (during Period 1) or 0.1 gm. of dried mixed grasses (during Period 2) was fed daily in addition to a diet deficient in fat-soluble vitamin. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet.

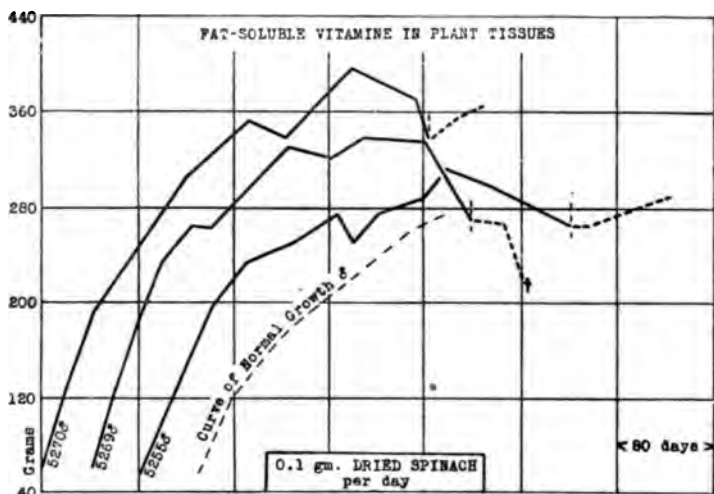


CHART IX. Experiments in which 0.1 gm. of dried spinach was fed daily in addition to a diet deficient in fat-soluble vitamin. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet. Rat 5269 died of lung disease.

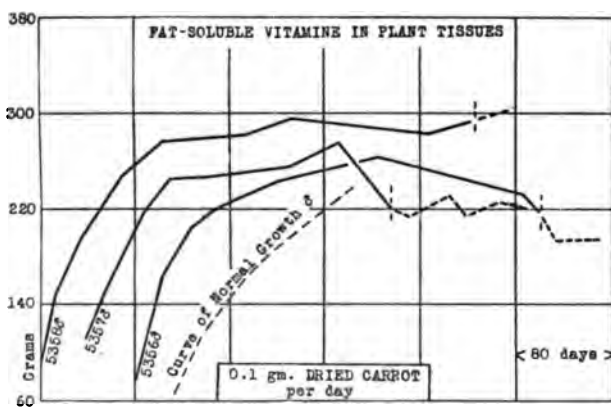


CHART X. Experiments in which 0.1 gm. of dried carrot was fed daily in addition to a diet deficient in fat-soluble vitamin. The interrupted lines indicate the period during which 18 per cent of butter fat was included in the diet.

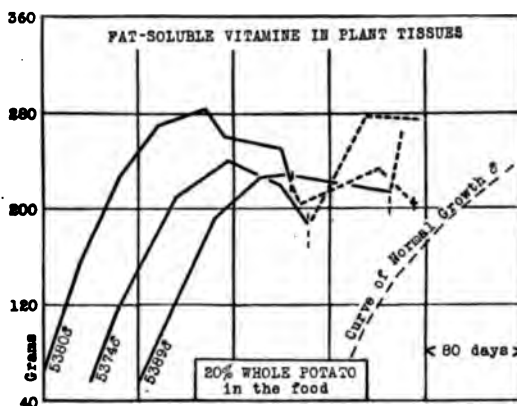


CHART XI. In these experiments the food consisted of

	per cent
Meat residue.....	15
Whole potato.....	20
Salt mixture*.....	4
Starch.....	36
Lard.....	25
Yeast.....	0.2 gm. daily.

* The composition of the salt mixture is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The fat-soluble vitamine was furnished by the dried potato until the period represented by the interrupted line, during which 0.5 gm. of butter fat per day was fed in addition. Rat 5380 died of lung disease.



STUDIES OF ACIDOSIS.

XVI. THE TITRATION OF ORGANIC ACIDS IN URINE.

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(Received for publication, February 24, 1920.)

Description of Method.

100 cc. of urine, roughly measured, are thoroughly mixed with 2 gm. of finely powdered calcium hydroxide, allowed to stand about 15 minutes with occasional stirring, and then passed through a dry folded filter. This treatment removes carbonates and phosphates. To 25 cc. of the filtrate in a 125 to 150 cc. test-tube of clear glass¹ one adds 0.5 cc. of 1 per cent phenolphthalein solution, and 0.2 N hydrochloric acid from a burette (amount need not be measured) until the pink color just disappears (pH = approximately 8). 5 cc. of 0.02 per cent tropeolin OO solution are then added. As the indicator solution is added it is thoroughly mixed with the urine by shaking the tube; if this precaution is omitted some of the tropeolin OO may be precipitated. Finally 0.2 N hydrochloric acid is added from the burette until the red color equals that of a standard solution containing 0.6 cc. of 0.2 N HCl, 5 cc. of tropeolin OO solution, and water to a total volume of 60 cc. When the end-point is approached, sufficient water is added to the titrated solution to make its volume equal to that of the 60 cc. standard solution used in a similar tube as a color control.

In comparing the color of the titrated solution with that in the standard, it is convenient during the titration to hold the two

¹ We use the tubes of Pyrex glass made by the manufacturers for urea determinations by the Van Slyke and Cullen technique. The tubes are 30 mm. inner diameter, 200 mm. long, and uniform in size.

tubes side by side between the thumb and fingers, the tube containing the urine being the one held nearer to the tips of the fingers where it can be easily shaken as the 0.2 N acid is run in from the burette.

Sometimes it is desirable to use a similar technique for the phenolphthalein end-point also. In this case a tube of urine filtrate to which no phenolphthalein is added serves as a standard.

We have found that as the final end-point with tropeolin OO is approached comparison of colors is somewhat facilitated by placing the two tubes side by side in a comparator of the form described by Dernby and Avery, although with practice the end-point may be located within 0.1 cc. by merely holding the tubes together as described above.

Calculation.

From the volume of 0.2 N HCl used to titrate from the end-point of phenolphthalein to that of the tropeolin OO, the amount, usually 0.7 cc., is subtracted which is utilized in a similar titration of a control determination in which water is substituted for the urine. The volume of 0.2 N HCl thus corrected represents the approximate organic acid content of the urine sample, plus the creatine and creatinine, and an amount of amino-acids ordinarily negligible.

In order to calculate the results in terms of cc. of 0.1 N organic acid per liter, the figure representing the cc. of 0.2 N HCl used in the titration is multiplied by 80 (by $\frac{1,000}{25} = 40$ in order to transfer figure from 25 cc. to 1,000 cc. of urine, and by 2 to change from 0.2 N to 0.1 N terms).

Correction for Creatinine.—A 0.1 M solution of creatinine (11.32 mg. per cc.) titrates in the above determination as a 0.1 N solution of organic acid. Therefore, in order to correct for the creatinine, the cc. of 0.1 N organic acid per liter calculated from the above titration may be diminished by

$\frac{\text{mg. creatinine per liter urine}}{11.32}$ or by $\frac{\text{mg. creatinine N per liter urine}}{4.2}$.

The simplest way is to subtract the creatinine correction directly from the cc. of 0.2 N acid used in the titration, and mul-

tiply the difference by 80. In this case the correction is $\frac{1}{80}$ as great as the above; *i.e.*, cc. correction =

$$\frac{\text{mg. creatinine per liter urine}}{906} \text{ or } \frac{\text{mg. creatinine N per liter urine}}{336}$$

Example.—

0.2 N HCl used in titration	cc.
Correction found in blank analysis.....	7.6
Creatinine correction for 500 mg. creatinine N per	
liter urine. Correction = $\frac{500}{336}$ cc. =	1.2 "
Total correction	1.9 cc.
Corrected titration figure = 7.6 - 1.9.....	5.7
0.1 N organic acid per liter = 80 × 5.7.....	456.0

Tropeolin OO was preferred by us as indicator for the final end-point. In neutral solution it gives nearly the same yellow color as urine, but so much more intense that a water solution of 0.002 N hydrochloric acid with the indicator can be used as a color standard without the use of a comparator. Very dark urines may need greater dilution, but such are not often encountered. Another advantage of this indicator is that its maximum acid color is not reached even at pH 2.7, so that if too much HCl is added in the titration the solution becomes redder than the standard. This particular advantage is possessed in much less degree by the three indicators mentioned below as alternatives.

Other indicators that may be used are methyl orange, tetrabromophenolsulfonephthalein (bromophenol blue, Clark and Lubs), and dimethylaminoazobenzene. To some eyes the color change of one of these dyes may be more readily detected than that of tropeolin OO. The two azo dyes are not much different in color from tropeolin OO, both changing from yellow to red, but the bromophenol blue turns from blue to a clear yellow on acidifying, and affords a very different alternative. To the authors the tropeolin OO end-point appeared the most satisfactory, however.

Theoretical Basis of Method.

The method is based on the following previously known facts:

1. Relatively little strong mineral acid is required to change the hydrogen ion concentration of a water solution from 10^{-8} to 2×10^{-3} if the only electrolytes present are alkali salts of strong acids, such as sulfates and chlorides.

2. If the salt of a weak acid is present, however, the addition of nearly a full molecule of hydrochloric acid for each molecule of such salt is necessary in order to cause the above change in hydrogen ion concentration. The organic acids known to occur in normal and pathological urines, in amounts sufficient to be quantitatively significant in the total acid excretion of the body, belong to the class of weak acids whose salts behave in the above manner.

3. The only mineral acids found in significant amounts in urine which belong to the class of weak acids, and therefore form salts which show the above behavior, are phosphoric and carbonic acids.

4. Very weak bases form salts which behave like those of the weak acids. Creatinine is titrated almost quantitatively in changing the hydrogen ion concentration from 10^{-3} to 2×10^{-3} , and creatine to about 60 per cent. Aside from the traces of amino-acids, these appear to be the only bases of this kind present in considerable amount in human urine.

Effect of the Different Organic Acids of the Urine on the Titration.

The titration figure obtainable by titrating between two hydrogen ion concentrations a solution containing the salt of an acid of a known dissociation constant may be calculated as follows:

From the law of mass action:

$$(1) \text{H}^+ = k \frac{HA}{A'}$$

H^+ = hydrogen ion concentration in terms of normality.

A' = anion of acid.

k = dissociation constant of the acid.

HA = free, undissociated acid.

BA = salt of the acid.

λ = degree of dissociation of the salt into Na and Ac.

When the salt of the acid is present, and dilutions are of the magnitudes used in titrations (0.1 to 0.01 M), the equation becomes practically

$$(2) \quad H^+ = \frac{k}{\lambda} \times \frac{HA}{BA}$$

As λ in the high dilutions encountered approaches unity, it may in approximate calculations be neglected.

Equation 1 may then be expressed as

$$(3) \quad H^+ = k \times \frac{HA}{BA} \text{ or } \frac{HA}{BA} = \frac{H^+}{k}$$

For acetic acid $k = 1.8 \times 10^{-5}$.

When pH equals 8, or $H^+ = 1 \times 10^{-8}$, we therefore have in the case of acetic acid $\frac{HC_2H_3O_2}{BC_2H_3O_2} = \frac{10^{-8}}{1.8 \times 10^{-5}} = \frac{1}{1,800}$. One part in 1,801 parts, or 0.05 per cent, of the acid is free.

When pH = 2.7, $H^+ = 2 \times 10^{-3}$, and we have $\frac{HC_2H_3O_2}{BC_2H_3O_2} = \frac{2 \times 10^{-3}}{1.8 \times 10^{-5}} = \frac{200}{1.8}$.

At a pH of 2.7, therefore, $\frac{200}{201.8}$, or 99.2 per cent, of the acid is free. Changing the hydrogen ion concentration of an acetate solution from the slightly alkaline reaction of 10^{-8} N (or a pH of 8) to the acid reaction of 2×10^{-3} (pH = 2.7) approximately the reaction of 0.002 N HCl) therefore requires an amount of HCl equal in molecular equivalents to 99.15 per cent of the total acetate present.

For the different acids which occur or may occur in human urine, the values in Table I are calculated. The values of the constants are for 25° unless otherwise indicated.

Comparison of the results so calculated with those experimentally obtained in titrating solutions of some of these acids is satisfactory, as shown in Tables III, IV, and VI of the experimental part of this paper. The conclusion seems justified that the titration as carried out estimates certainly over 90 per cent of the organic acids of the urine, and presumably over 95 per cent, since

a higher titration value by 3 or 4 per cent is obtained for those acids excreted as ammonium salts. The data for carbonic and phosphoric acids indicate the necessity for their removal before the organic acids are titrated.

TABLE I.
Calculated Titration Values of Weak Acids of the Urine.

Acids.	Dissociation constant.	Acid free at		Calculated proportion of acid determined by titrating from pH 8 to pH 2.7.
		$H^+ = 10^{-8} N$ pH = 8	$H^+ = 2 \times 10^{-3}$ pH = 2.7	
		per cent	per cent	per cent
<i>Organic.</i>				
Uric*	1.5×10^{-6}	0.5	99.9	99.4
Acetic†	1.8×10^{-5}	0.0	99.2	99.2
β -hydroxybutyric†	2.0×10^{-5}	0.0	99.0	99.0
Lactic†	1.4×10^{-4}	0.0	93.5	93.5
Acetoacetic†	1.5×10^{-4}	0.0	93.1	93.1
Citric§	2.0×10^{-4}	0.0	91.0	91.0
Formic*	2.1×10^{-4}	0.0	90.6	90.6
Hippuric†	2.2×10^{-4}	0.0	90.2	90.2
<i>Mineral.</i>				
$H(NaHPO_4)_{ }$	2.0×10^{-7}	2.5	100	97.5
$H(HCO_3)_{ }$	3.5×10^{-7}	4.2	100	95.8

* His and Paul.

† Ostwald.

‡ Henderson and Spiro.

§ Shown by Amberg and McClure to occur in amounts equivalent to 60 to 70 cc. of 0.1 N acid in a normal 24 hour urine. The titration values for citric acid given in Column 5 are those directly determined by Sørensen. The constant is estimated from them.

|| Sørensen.

¶ Kendall.

Effect of Weak Bases of the Urine on the Titration.

The amount of strong acid required to change the pH of a solution of a weak base from 8 to 2.7 may be calculated from the dissociation constant K_b .

$$K_b = OH' \times \frac{\text{salt of base}}{\text{free base}} = \frac{10^{-14}}{H^+} \times \frac{\text{salt of base}}{\text{free base}}$$

The "salt of base" represents the amount combined with acid. At pH 8, therefore, salt of base, or $\frac{\text{acid combined with base}}{\text{free base}} = K_b \times \frac{10^{-8}}{10^{-14}} = K_b \times 10^6$. At pH 2.7, or $H^+ = 2 \times 10^{-3}$, the ratio is $K_b \times \frac{2 \times 10^{-3}}{10^{-14}} = 2 K_b \times 10^{11}$. The difference between the acid bound by a given base at pH 8 and that bound at pH 2.7 represents the amount required to titrate between the two points. Table II contains a list of the weak bases of the urine, with their constants and the proportion of an equivalent of HCl

TABLE II.
Calculated Titration Values of Organic Bases of the Urine.

Base.	Basic dissociation constant. K_b	Base free at		Proportion estimated by titrating with HCl from $H^+ = 10^{-8}$ to $H^+ = 2 \times 10^{-3}$	
		$H^+ = 10^{-8}$	$H^+ = 2 \times 10^{-3}$	Calculated.	Observed.
		per cent	per cent	per cent	per cent
Urea*.....	0.0015×10^{-11}	100	99.7	0.3	0.2
Creatinine†..	1.81×10^{-11} *	100	24.0	76.0	99
Creatine‡....	3.57×10^{-11} *	100	12.3	87.7	60
Ammonia‡....	1.5×10^{-8} †	6.2	0.0	6.2	5.3-6.0

* Walker and Wood.

† Measured at 40°, Wood.

‡ Noyes, Kato, and Sosman.

required to titrate each from pH 8 to pH 2.7, calculated as above indicated. The constants are from data obtained at 25°, except for creatine and creatinine. In Column 5 results are brought forward from Table V, showing the amounts of HCl bound by the different bases in the titration, as determined experimentally.

Urea is, both by observation and calculation, practically without effect on the results of the titration, even when the urea concentration is at the maximum observed in human urine.

The available data on the K_b of creatine and creatinine do not yield calculated results corresponding so closely with those experimentally obtained as do the data on the other substances requir-

ing consideration. The divergence is perhaps due to the fact that Wood's values for K_b of creatine and creatinine were determined at 40°, while the titration is performed at 20°. It is evident, however, that practically all the creatinine is titrated as organic acid. The amount of this substance excreted varies between 13 and 27 mg. per kilo of body weight per 24 hours (Folin, 1905). The mean, 20 mg., would neutralize 1.8 cc. of 0.1 N acid per kilo or 108 cc. for a 60 kilo individual.

Creatine when present titrates to about 60 per cent as an organic acid; but it is excreted by adults only in conditions involving rapid autolysis of muscle tissue, and would therefore not, as a rule, require consideration.

Ammonia is titrated to the extent of 5 to 6 per cent, but the actual effect of the presence of organic acids as ammonium rather than fixed alkali salts is to make the results of the titration with most of the acids approximate more closely the theoretical values, as shown in Table IV. The ammonium salts of the organic acids titrate 2.3 to 4.6 per cent more completely than the sodium salts, not 6.2 per cent more completely, as would be theoretically expected, and as is approximately realized for the ammonium salts of hydrochloric and sulfuric acids. The observed positive ammonia error is such as to make the results obtained with all but the weakest organic acids approximate more closely to 100 per cent than the results obtained in the absence of ammonia. The tendency of the ammonia error to correct the opposite error in the organic acid titration is enhanced by the fact that ammonia and organic acid excretion tend to run parallel, particularly when acid excretion is abnormally high, as in diabetic acidosis. For the reasons, therefore, that the ammonia correction is not great and is of a nature actually to diminish, as a rule, the other error in the determination, it has seemed not only simpler but better to attempt no correction for it in urine analyses.

Effect of Amino-Acids on the Titration.

Amino-acids if present in large amount would be disturbing factors, as at an H^+ of 2×10^{-3} they bind with their NH_2 groups considerable amounts of acid. Glycocoll, which does not differ much from the other monoamino-acids in this respect, binds

about $\frac{1}{3}$ molecule of HCl at this H^+ . The amount is calculated as follows:

The acid constant for glycocoll is 3.4×10^{-10} , the basic constant 2.9×10^{-12} , as calculated by Winkelblech from conductivity measurements. From the acid constant we have by calculating as above:

$-\text{COOH}$ free at $H^+ = 10^{-8} N$	$-\text{COOH}$ free at $H^+ = 2 \times 10^{-8} N$	Proportion of COOH group estimated by titration from $H^+ = 10^{-8}$ to $H^+ = 2 \times 10^{-8}$
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
96.7	100	3.3

The function of the NH_2 group is similarly calculated from the basic constant, $K_b = 2.9 \times 10^{-12} = (\text{OH})' \times \frac{\text{glycine chloride}}{\text{free glycine}}$ or

$$\frac{\text{COOH-CH}_2\text{-NH}_2}{\text{COOH-CH}_2\text{-NH}_2 \text{ HCl}} = \frac{(\text{OH})'}{2.9 \times 10^{-12}} = \frac{10^{-14}}{H^+ \times 2.9 \times 10^{-12}} = \frac{10^{-2}}{H^+ \times 2.9}$$

From these values we calculate:

$-\text{NH}_2$ free at $H^+ = 10^{-8} N$	$-\text{NH}_2$ free at $H^+ = 2 \times 10^{-8} N$	Proportion of NH_2 group estimated by titration with HCl from $H^+ = 10^{-8}$ to $H^+ = 2 \times 10^{-8}$
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
100	63.3	36.7

The total consumption of HCl by both COOH and NH_2 groups in the titration should be, according to the above calculation, $0.03 + 0.367 = 0.40$ molecule of HCl per 1 molecule of glycine present. The actual amount observed by Sørensen was 0.385 molecule.

The other monoamino-acids apparently bind similar amounts of HCl. The constants for leucine and alanine were determined by Winkelblech as follows: leucine, $K_a = 3.1 \times 10^{-10}$, $K_b = 2.7 \times 10^{-12}$; alanine, $K_a = 9.0 \times 10^{-10}$, $K_b = 3.8 \times 10^{-12}$. According to these, leucine would require in the titration 0.38 molecule of HCl; alanine 0.36, nearly the same as glycocoll. The results in Table VII for the mixture of all the monoamino-acids obtained from casein are in the same neighborhood (44 per cent).

The amino-acid nitrogen constitutes 1 to 2 per cent of the total urinary nitrogen (Van Slyke, 1913-14; Henriques). On a daily excretion of 14 gm. of nitrogen, 2 per cent would indicate 200 cc. of 0.1 M amino-acids. The neutralizing power of such an amount of amino-acids in the titration would be about 80 cc. of 0.1 N hydrochloric acid.

Our knowledge of the nitrogenous constituents of the urine indicates the presence of no weak bases, aside from those discussed, in quantities sufficient to affect markedly the organic acid titration under discussion, and the nitrogenous excretory products have been so thoroughly studied that it is unlikely that any quantitatively important substances with definitely basic properties have been overlooked.

It therefore appears that in titrating the 24 hour urine of an adult of average size for organic acids, as described in this paper, about 100 cc. of the 0.1 N organic acid estimated is in reality due to creatinine and creatine, 80 cc. or less to amino-acids, and the remainder to organic acids.

EXPERIMENTAL.

Titration of Organic Acids in Water Solutions.—A 20 cc. portion of each acid, of approximately 0.1 N concentration, was titrated in a 100 cc. test-tube with either 0.1 N sodium hydroxide or 0.1 N ammonium hydroxide to neutrality with 0.5 cc. of 1 per cent phenolphthalein. 1 cc. of 0.1 per cent tropeolin OO was then added, and the solution titrated back with 0.2 N HCl to pH 2.7, using 0.002 N HCl solution as standard. The results are given in Tables III and IV.

Titration of Weak Bases in Water Solutions.—Solutions of the bases in 25 cc. portions were brought to pH 8 by addition of 0.1 N NaOH or 0.2 N HCl until a barely visible pink color was reached; then tropeolin OO was added and the solution titrated to pH 2.7. The results are given in Table V.

Effect of Concentration of Phenolphthalein on its End-Point in Presence of Ammonium Salts.—The concentration of phenolphthalein to some extent affects the pH at which the pink color is just visible. If there is but little indicator present a greater part of it must be in the colored form to give a perceptible pink

TABLE III.

Titration of Sodium Salts of Organic Acids.

Acid.	(A) 0.1 N NaOH to neutralise acid to phenol- phthalein.	(B) 0.2 N HCl to titrate back to pH 2.7 with tropaeolin OO.	(C) Average 0.2 N HCl corrected for blank.	(D) Organic acid determined. 200 (C) (A)	Organic acid theoreti- cally titrat- able from pH 8 to pH 2.7 (from Table I).
	cc.	cc.	cc.	per cent	per cent
Blank.....	0.1	0.50	0.00		
Acetic.....	20.00	10.60	9.95	99.5	99.4
		10.50			
Citric.....	19.86	9.30	8.88	89.4	91.0
		9.35			
Lactic.....	20.28	9.90	9.40	92.7	93.5
		9.90			
Hydrochloric.....	20.00	0.70	0.20	1.0	

TABLE IV.

Titration of Ammonium Salts of Organic Acids.

Acid.	(A) 0.1 N acid present.	(B) 0.1 N NH ₄ OH to neutral- ize acid to phenol- phthalein at pH 8.	(C) 0.2 N HCl to titrate back to pH 2.7.	(D) Average 0.2 N HCl corrected for 0.5 cc. blank.	(E) Proportion of organic acid de- termined 200 (D) (A)	Proportion of NH ₄ salt theo- retically titratable; i. e., that for acid calcu- lated in Table I + 6.2 per cent for NH ₄ present.	Differ- ences be- tween average percentage of Na salt and NH ₄ salt titrated.
	cc.	cc.	cc.	cc.	per cent	per cent	per cent
Acetic.....	19.68	20.51	10.70	10.25	104.1	105.6	4.6
	19.68	20.47	10.80				
Citric.....	21.04	21.55	10.30	9.79	93.0	97.2	3.5
	21.04	21.51	10.27				
Lactic.....	20.06	20.96	10.03	9.50	94.7	99.7	2.3
	20.06	20.96	10.03				

than when the total amount of indicator is greater. Consequently the amount of extra alkali required to make a solution of an ammonium salt show pink with phenolphthalein is some-

TABLE V.

Observed Behavior of Weak Bases when Titrated from pH 8 to pH 2.7.

Base.	Amount present in the 25 cc. of solution titrated.		0.2 N HCl required in titrating from pH 8 to pH 7.	Proportion of base titrated.	Proportion of base calculated as titratable from dissociation constant (Table II).
	gm.	cc. 0.2 N	cc.	per cent	per cent
Urea.....	1.000	83.3	0.1	0.12	0.3
Creatine.....	0.200	7.6	4.1	60.0	87.7
Creatinine.....	0.100	4.41	4.32	97.8	76.0
	0.200	8.83	8.80	99.7	
Monoamino-acids	0.100	7.37*	3.25	44.2	36.0-40.0 for glycine, leucine, and alanine.
from casein.....	0.200	14.63	6.37	43.5	
	0.200	14.63	6.29	43.0	
Ammonia (as	*	12.50	0.67	5.4	6.2
(NH ₄) ₂ SO ₄).....		12.50	0.75	6.0	
Ammonia (as		9.82	0.53	5.4	6.2
NH ₄ Cl).....			0.52	5.3	

* Calculated on a nitrogen content of 10.3 per cent. The preparation was made by hydrolyzing casein with sulfuric acid, precipitating the bases with phosphotungstic acid, and concentrating the filtrate to dryness under reduced pressure after the phosphotungstic and sulfuric acids had been removed.

TABLE VI.

Effect of Phenolphthalein Concentration on End-Point in Presence of Ammonium Salts.

0.05 M (NH ₄) ₂ SO ₄ .	1 per cent phenol- phthalein.	0.1 N NaOH to turn pink to phenol- phthalein.	0.2 N HCl to change from phenolphthalein end-point to pH 2.7.		Proportion of ammonia titrated from phenol- phthalein end-point to pH 2.7
			Uncorrected.	Minus 0.5 cc. for correction.	
cc.	cc.	cc.	cc.	cc.	per cent
25	0.1	0.85	1.42	0.92	7.3
25	0.2	0.65	1.36	0.86	6.9
25	0.5	0.45	1.20	0.70	5.6
25	1.0	0.45	Too cloudy with precipitated phenolphthalein to titrate.		

what dependent on the amount of indicator used. This is shown by the results in Table VI. It is desirable to use in performing the titrations 0.5 cc. of 1 per cent phenolphthalein solution, as directed, rather than the indefinitely measured drop or two which suffices in ordinary titrations.

TABLE VII.
Titration of Organic Acids Added to Urine.

Organic acid added.	0.1 N organic acid added to 100 cc. urine.	0.2 N HCl used in duplicate titrations of 25 cc. urine filtrate.	Average titration figure minus that for urine alone.	0.1 N added organic acid per liter diluted urine.		Proportion of added organic acid determined.
				Found.	Added.	
	cc.	cc.	cc.	cc.	cc.	per cent
Acetic.	0	3.00 3.00				
	25	4.55 4.53	1.54	123	125	98.4
	50	6.20 6.15	3.17	253	250	101.2
	100	9.15 9.10	6.13	490	500	98.0
Lactic.	0	2.87 2.87				
	25	4.25 4.20	1.36	109	117*	93.2
	50	5.50 5.60	2.68	214	236*	90.7
	100	8.30 8.25	5.41	432	472*	91.6

* The 0.1 N lactic acid used in this experiment had the factor 0.945.

Titration of Known Amounts of Organic Acids Added to Urine.—100 cc. portions of a mixed sample of normal urine were mixed with portions of 25, 50, and 100 cc. respectively of acetic or lactic acid. Each mixture was then diluted to 200 cc., and 100 cc. portions were treated as previously described for determination.

TABLE VIII.
Excretion of Organic Acids with Creatinine Correction.
 Data from hospital patients.

		Urine excretion.														
Subject.	Weight.	Condition.	Period.	Volume.	Creatinine N.			0.2 N HCl used in titrating from pH 8 to pH 2.7.		0.1 N organic acid content.						
					hrs.	cc.	gm.	cc. 0.1 M	Duplicates.	Average minus 0.6 cc. correction for blank.	Uncor- rected for creati- nine.	Correct- ed for creati- nine.	Total.	Per kilo.	Uncor- rected for creati- nine.	Correct- ed for creati- nine.
Z	60	Myocarditis, decompensation on admission.	12 (day) 12 (night) 24	658 946 1,604	0.240 0.261 0.501	57 62 119	7.0, 6.9 4.3, 4.1	6.35 3.60	334 272 606	277 210 487	cc. cc. cc.	cc.	cc.	cc.	cc.	cc.
O	60	Myocarditis, some decompensation.	12 (day) 12 (night) 24	707 744 1,451	0.223 0.216 0.439	53 51 104	5.1, 5.25 5.0, 5.0	4.57 4.40	258 262 520	205 211 416	cc. cc. cc.	cc.	cc.	cc.	cc.	cc.
C	55	Chronic aortic endocarditis.	12 (day) 12 (night) 24	242 332 574	0.141 0.195 0.336	34 46 80	15.1, 15.3 12.8, 12.9	14.60 12.25	282 325 607	248 279 527	cc. cc. cc.	cc.	cc.	cc.	cc.	cc.
D	62	Chronic myocarditis with decompensation.	12 (day) 12 (night) 24	647 750 1,397	0.226 0.196 0.422	54 47 101	5.6, 5.7 4.4, 4.3	5.05 3.75	261 225 486	207 178 385	cc. cc. cc.	cc.	cc.	cc.	cc.	cc.
H	50	Orthostatic albuminuria.	12 (day) 12 (night) 24	364 380 744	0.091 0.091 0.182	22 22 44	5.55, 5.55 5.25, 5.10	4.95 4.48	144 136 280	122 114 236	cc. cc. cc.	cc.	cc.	cc.	cc.	cc.

- The results are given in Table VII. The results are essentially the same as those obtained with acetic and lactic acids in pure water solutions.

Organic Acid Excretion by Individuals with Normal Metabolism.

The data given are sufficient only to indicate the usual excretion of organic acids; the possible normal variations, particularly under unusual conditions, may be greater. The figures of Table

TABLE IX.
24 Hour Excretion of Organic Acids by Normal Young Men.

Subject.	Weight.	24 hour urine.			
		Volume.	0.1 N organic acids uncorrected for creatinine.*		Total N.
			cc.	cc. per kg.	
	kg.	cc.	cc.	cc. per kg.	gm.
Ce.....	54.4	1,000	492	9.0	9.3
Dy.....	68.0	1,650	657	9.8	11.5
E.....	68.0	975	583	8.5	11.7
Fr.....	62.1	1,500	531	8.5	13.2
H.....	68.0	1,150	412	6.1	7.8
Sh.....	56.6	1,500	453	8.0	10.0
K.....	68.4	1,000	490	7.2	8.7
Sp.....	57.2	1,400	521	9.1	9.0
Fe.....	82.6	1,100	748	9.1	15.5
Dn.....	87.0	1,300	493	5.7	13.2
Ch.....	56.2	1,100	420	7.5	11.2
K.....	61.2	700	499	8.2	10.0
Ck.....	56.6	1,300	547	9.7	12.1
Average.....				8.2	

* The creatinine correction would reduce the total organic acid figure by about 2 cc. per kilo.

VIII are from afebrile heart patients, with apparently normal metabolism. The day periods are from 6 a.m. to 6 p.m., the night periods from 6 p.m. to 6 a.m. The data of Table IX are from a series of healthy young men. The figures indicate that the usual excretion of organic acids uncorrected for creatinine varies from about 280 to 750 cc. of 0.1 N acid per 24 hours, or from 5.6 to 11 cc. per kilo of body weight. The creatinine correction reduces the figures to 240 to 600 and 4.7 to 9.6 cc. respectively.

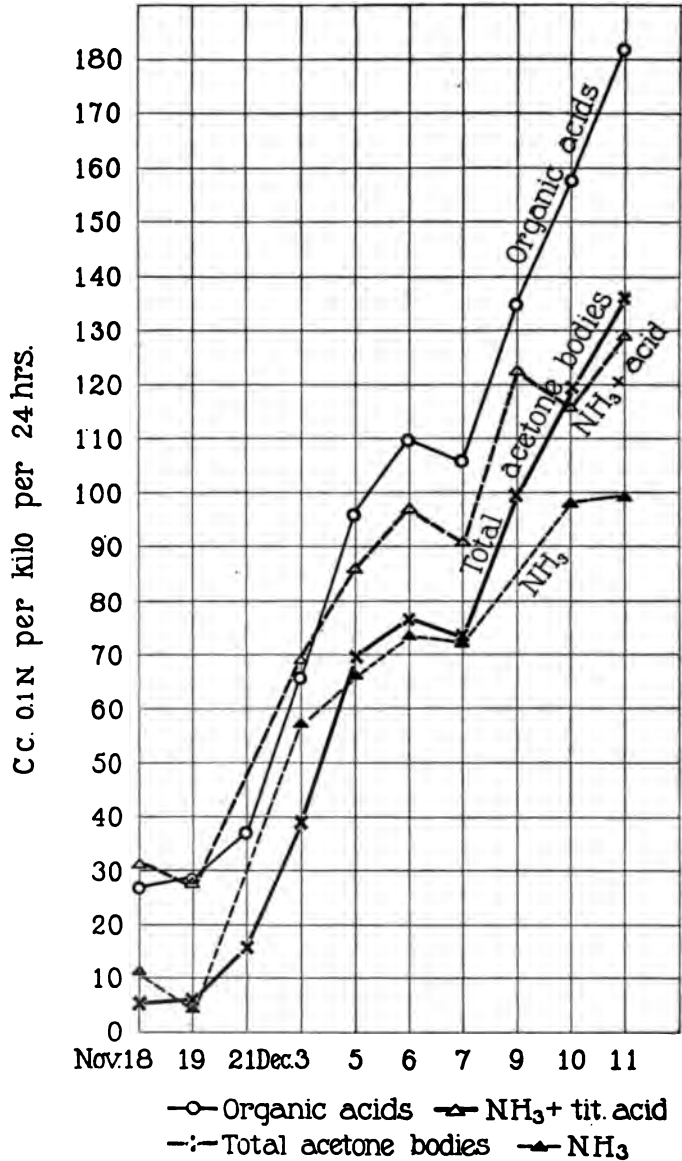


FIG. 1. Excretion in a case of diabetic acidosis.

*Comparison of Total Organic Acid Excretion with Acetone Bodies
Excretion in Diabetes.*

The data given in Fig. 1 were obtained with the only case of diabetic acidosis which we have studied since the organic acid titration method has been available. Although all the data are from one case, they nevertheless represent every stage of diabetic acidosis, from the time when it was slight, with little ketonuria, up to the point of coma, with tremendous ketonuria. The patient was a child of 2 years, weighing 8 kilos. The organic

TABLE X.

Organic Acid Excretion in a Non-Fatal Case of Methyl Alcohol Poisoning.

Date.	CO ₂ bound as bicarbonate by 100 cc. of plasma.	Excretion per liter urine.						
		Creatine.	Creatinine.	Total 0.1 N organic acids.*	0.1 N acetone bodies.	0.1 N lactic acid.	0.1 N formic acid.	Under- determined 0.1 N organic acids.
1919	cc.	gm.	gm.	cc.	cc.	cc.	cc.	cc.
Nov. 24.....	36.4	0.202	0.558	2,042		173	274	1,595
" 25.....	36.0	0.283	1.000	2,076	481	83		1,512
" 26.....	86.2	0.535	0.800	1,377	143	30	130	1,074
" 29.....	76.7	0.300	0.590	262				
Dec. 1.....		0.180	0.538	129				
" 2.....		0.137	0.557	141				
" 3.....				220†				
" 11.....		0.105	0.378	86				
" 17.....		0.024	0.476	138				
" 20.....								

* Corrected for creatine and creatinine.

† " " " " " estimated not determined.

acid figures recorded in Fig. 1 are not corrected for creatine and creatinine, so that they are higher than, though parallel to, the actual organic acid excretion. The "total acetone bodies," β -hydroxybutyric acid, acetoacetic acid, and acetone were determined by the gravimetric method of Van Slyke (1917), the ammonia as described by Van Slyke and Cullen, and the titratable acid by the method of Folin (1903).

It is evident from the chart that the organic acids of the urine, determined by the technique outlined above, paralleled the ace-

tone body excretion with a high degree of accuracy through all stages of the acidosis, the parallelism being more accurate than that of the ammonia, or even the ammonia plus titratable acid.

It appears that the rise above the normal output in organic acid excretion may be used as an approximate measure of the acetone body excretion in diabetes, the determination of organic acids being as simple as that of ammonia and less influenced by other factors, such, in particular, as alkali administration.

Organic Acid Excretion in Methyl Alcohol Poisoning.—The data of Table X illustrate an acidosis caused by organic acids other than the familiar acetone bodies. The data represent some preliminary work on methyl alcohol poisoning and are inserted here only for their interest in illustrating a hitherto unfamiliar type of acidosis.

SUMMARY.

The organic acids present both free and as salts in urine are estimated by titrating between the hydrogen ion concentrations represented by pH 8 and pH 2.7 respectively, after removal of phosphates and carbonates by means of calcium hydroxide. It appears that the titration represents between 95 and 100 per cent of the organic acids present. It also includes weak bases whose dissociation constants fall within a range in the neighborhood of 10^{-11} , but of this class only creatinine, and at times creatine, appear to be present in significant amounts in human urine.

The average 24 hour excretion of organic acids in thirteen healthy young men was, per kilo of body weight, 8.2 cc. of 0.1 N acid uncorrected for creatinine, or approximately 6 cc. corrected for creatinine; the extreme range was from 5.7 to 9.8 cc. uncorrected for creatinine. There appears to be little difference between day and night periods in rate of organic acid excretion.

Data from cases of methyl alcohol poisoning and diabetes respectively are given as examples of acidosis due to organic acids of different types. In the case of methyl alcohol poisoning part of the total organic acid excretion was due to formic, lactic, and hydroxybutyric acids, but the greater part to acids of unknown nature.

In the case of diabetes, which progressed to coma, the rise in acetone body excretion was accurately paralleled by the rise in

the titrated organic acids. The parallelism was so close as to indicate the probabilities (1) that organic acids other than the acetone bodies are not excreted in significant amounts in diabetic acidosis, and (2) that the easily performed organic acid titration may be used for approximate estimation of the acetone bodies in diabetic urine.

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DETERMINATION OF THE FIBRIN, GLOBULIN, AND ALBUMIN NITROGEN OF BLOOD PLASMA.

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An investigation into the distribution of the plasma nitrogen, which formed part of a study of the fate of the protein digestion products, led us to develop a technique for the determination of the plasma proteins which gives consistent results and requires no special apparatus. It is based entirely on Kjeldahl determinations, of which the following four are required: (1) total nitrogen of the plasma, (2) fibrin nitrogen, (3) filtrate nitrogen (filtrate, containing albumin and non-protein nitrogen, obtained after precipitating the globulins by half saturation with ammonium sulfate), and (4) non-protein nitrogen.

In the technique finally arrived at the fibrin was precipitated by calcium chloride under definite conditions from plasma containing 0.5 per cent of potassium oxalate, was washed free from other nitrogenous substances, and determined by Kjeldahl.

The globulin was precipitated (together with the fibrin) by the usual half saturation with ammonium sulfate. The nitrogen of the filtrate was determined by Kjeldahl, after removal by distillation of the ammonia of the ammonium sulfate. For the distillation it was found necessary to standardize the conditions accurately in order to make the removal quantitative and at the same time avoid splitting off labile nitrogen from proteins. The non-protein nitrogen was determined on a separate sample of plasma.

The fibrin, globulin, and albumin are calculated as follows:

Fibrin N, determined directly.

Globulin N = Total N - (filtrate N + fibrin N)

Albumin N = Filtrate N - non-protein N

There are two steps in the determination of the albumin and globulin contents of plasma; the first is the separation of the two proteins, or groups of proteins, by precipitation of the globulin with salt, either saturated magnesium sulfate, or half saturated ammonium sulfate. Robertson has reviewed the work on globulin precipitation, and is convinced that ammonium sulfate is the most satisfactory salt for the purpose. We have, therefore, utilized ammonium sulfate precipitation from the start, and since the results have been uniformly consistent have not experimented with other globulin precipitants.

The second step is the determination of the proteins after they have been separated. By different authors this has been done by weighing, by nitrogen determination after dialysis to remove ammonium salts, by the nephelometric method, or by the use of the refractive indices of the proteins as developed by Reiss and by Robertson. The errors inherent in washing and weighing the globulin precipitate are too great to allow accurate results. With nephelometric determinations we have not been able to obtain the desirable degree of accuracy, and a proper refractometer was not available at the time the work was done. We consequently were led to develop a technique in which all the final determinations were made by the Kjeldahl method.

Description of Methods.

Fibrin Determination.

To 5 cc. of plasma, from blood to which 0.5 per cent of potassium oxalate has been added, add 150 cc. of 0.8 per cent NaCl and 5 cc. of a calcium chloride solution containing 2.5 gm. of anhydrous CaCl_2 per 100 cc. Allow complete coagulation to occur (10 to 15 minutes) and filter through filter paper. Wash with 0.8 per cent NaCl five times, allowing each washing to remain in contact with fibrin for 10 minutes by closing the outlet of the funnel for that period. Transfer filter paper containing fibrin clot to Kjeldahl flask and add 20 cc. of sulfuric acid, 12 gm. of potassium sulfate, and a crystal of copper sulfate, and determine nitrogen in the usual manner.

Albumin Determination.

Precipitation of Globulin.—To 5 cc. of plasma add 20 cc. of water and 25 cc. of saturated ammonium sulfate solution, allow to stand over night, and filter through a dry filter.

Removal of Sulfate Ammonia.—Place 20 cc. of filtrate (= 2 cc. of plasma) in a 500 cc. Kjeldahl flask, add 300 cc. of 50 per cent alcohol, 3 gm. of MgO (Merck's reagent), and 1 cc. of white mineral oil. Distill until distillate gives a negative test with red litmus paper.

Digestion of Residue.—To residue add 25 cc. of concentrated H_2SO_4 , 5 gm. of K_2SO_4 (addition of more, with magnesium sulfate present, would cause bumping), and a small crystal of copper sulfate. Digest to a light brown color. Then wash flask down with a few cc. of water and add 10 cc. more of H_2SO_4 . Continue digestion over a low flame for about 3 hours. Distill into N/14 HCl in the usual manner. Calculate nitrogen as "filtrate nitrogen."

Albumin nitrogen = Filtrate nitrogen - non-protein nitrogen

Total Plasma Nitrogen Determination.

The total nitrogen determinations are carried out on 2 cc. of plasma by the regular Gunning-Kjeldahl method, using 20 cc. of concentrated H_2SO_4 , 12 gm. of K_2SO_4 , about 0.2 gm. of copper sulfate, and digesting 3 hours after clearing.

Non-Protein Nitrogen Determination.

The non-protein nitrogen is determined in the filtrate obtained by precipitation of the plasma protein in 9 volumes of 2.5 per cent trichloroacetic acid (Greenwald, 1915). A 50 or 100 cc. measuring flask is half filled with the trichloroacetic acid solution, to which 5 or 10 cc. of plasma are added. The flask is then filled to the mark with the trichloroacetic acid solution, and the contents are thoroughly mixed. After standing 1 hour the contents of the flask are filtered through a dry filter, the filtrate is measured, and transferred to a Kjeldahl flask. 20 cc. of H_2SO_4 , 12 gm. of K_2SO_4 , and a crystal of copper sulfate are added, and the nitrogen is determined in the usual manner.

On the basis of Greenwald's recent results (1918), it would seem slightly preferable to use 5 per cent rather than 2.5 per cent trichloroacetic acid. The differences introduced are so minute, however, that for the determination of the proteins they are not significant.

Correction for Reagents.

It is necessary to determine the corrections for all the reagents. Our blanks averaged 0.46 cc. of $N/14$ HCl, a rather high value, but one constant for the given lot of reagents.

EXPERIMENTAL.

Determination of Filtrate Nitrogen.

In order to determine the albumin and non-protein plasma nitrogen in the filtrate from the globulin, it was necessary to find conditions for distilling off the ammonia of the ammonium sulfate without splitting off ammonia from any of the plasma proteins. In order to avoid such decomposition it was desirable to use in the distillation as weak an alkali as possible. Magnesium oxide, in former work on protein analyses (Van Slyke), had been found to be as mild an alkali as could be successfully used to drive off ammonia, and it proved to be suitable in this case also when used together with alcohol. It was found that the physical properties of the oxide were of importance. Tremendous bumping, resulting in broken flasks, took place with all but one brand of MgO. When Merck's reagent oxide was used with the addition of 1 cc. of white mineral oil, and the flask with its contents was frequently shaken until the boiling commenced, the distillation proceeded smoothly and without bumping.

Distillation with Water.—2 cc. of plasma and 10 cc. of saturated ammonium sulfate solution were diluted with 200 cc. of water in a 500 cc. Kjeldahl flask and an excess of magnesium oxide, 2 to 3 gm., was added (at 20°C. a half saturated ammonium sulfate solution contains 38 gm. per 100 cc. of the solution; 10 cc. would then require 1.7 gm. of MgO). The water and ammonia were distilled off. The nitrogen in the residue was then determined as outlined below.

In each case distillation was continued until moistened red litmus paper held in the distillate no longer turned blue at once. Actual cessation of ammonia distillation did not occur, because of a slight but continuous splitting off of ammonia from the proteins. Consequently if the litmus paper was held in the distillate for 2 minutes, an alkaline reaction could be obtained at any stage of the distillation. The end-point was therefore taken as the stage at which the distillate failed to turn litmus at once.

It was found that frequently the ammonia was not completely removed by distilling nearly to dryness once; it was necessary

TABLE I.

Albumin Determination. Removal of Ammonium Sulfate Nitrogen by Water Distillation in Presence of Magnesium Oxide.

2 cc. plasma + 10 cc. saturated $(\text{NH}_4)_2\text{SO}_4$ solution. • Total nitrogen of plasma controlled on 2 cc. duplicates.

Method of concentration.	MgO	H ₂ O used.	Final volume.	Proportion of plasma nitrogen recovered.	
				cc.	per cent
Distillation from Kjeldahl flask.	gm. 2.3	cc. 400 in two portions.	50		98.3
“ “ “ “	2.3	400 “ “ “	50		97.8
“ “ “ “	2.3	400 “ “ “	50		96.0
“ “ “ “	2.3	400 “ “ “	50		97.8
“ “ “ “	2.3	400 “ “ “	50		96.0
“ “ “ “	2.3	400 “ “ “	50		96.0
“ “ “ “	2.3	400 “ “ “	50		93.0
“ “ “ “	2.3	400 “ “ “	50		94.0
“ “ “ “	2.3	400 “ “ “	50		92.0

to add a second 200 cc. of water and distill again. With two distillations all the sulfate ammonia was removed, but with it from 2 to 8 per cent of the plasma nitrogen was lost, apparently as the result of ammoniacal decomposition of the plasma proteins (Table I). Regulation of the rate of distillation, of the final volume, etc., all failed to prevent this loss.

Distillation with Alcohol.—In attempting to reduce both time and temperature of distillation, a mixture of alcohol and water was substituted for the water. A few of the results are given in Table II.

It is evident that with the use of 50 per cent alcohol, 99 per cent of the plasma nitrogen can be consistently recovered. One distillation only, taking 30 to 45 minutes, is required to drive off all ammonia. In studying the distribution of the plasma proteins following digestion, the method was tested several times on plasma from each of a dozen dogs. Between 99.8 and 98.5 per cent of the plasma nitrogen was invariably recovered.

TABLE II.

Albumin Determination. Removal of Ammonium Sulfate Nitrogen by Distillation in Presence of MgO and Alcohol.

2 cc. plasma + 10 cc. saturated $(\text{NH}_4)_2\text{SO}_4$ solution.

MgO	H ₂ O	95 per cent alcohol.	Final volume.	Proportion of plasma nitrogen recovered.
gm.	cc.	cc.	cc.	per cent
3.5	150	150	25	99.6
3.0	150	150	25	99.2
2.5	150	150	15	99.6
3.0	150	150	Dry.	97.9
3.0	150	150	25	99.0
3.0	150	150	25	99.6
3.0	150	150	25	99.1
3.0	150	150	25	99.2
3.0	150	150	25	99.0

Fibrin Determination.

Dilution of Plasma.—If calcium is added to undiluted oxalated plasma, the entire mass jellies. If, however, the plasma is diluted with isotonic NaCl solution, the fibrin forms as a delicate membrane which contracts upon shaking or stirring to a small compact mass. Moreover, if the plasma is diluted ten- to thirtyfold the quantity of nitrogen in the solution adhering to the small clot is presumably much smaller than if the fibrin is whipped from undiluted plasma. In order to ascertain the best dilution, oxalated plasma was mixed with varying amounts of isotonic NaCl solution, which, to prevent globulin precipitation, was used instead of water. Preliminary experiments had shown that at least 2 molecules of calcium chloride should be added for each molecule of potassium oxalate present in the plasma.

The time required to complete the formation of the fibrin clot and the appearance of the clot were noted. Typical results are shown in Table III.

The clots formed when the plasma was diluted with 10 volumes of salt solution did not appear so satisfactory as those formed with either 20 or 30 volumes. There was no choice between

TABLE III.

Determination of Dilution Most Suitable for Plasma Fibrin Coagulation.

1 cc. plasma diluted as indicated.

Plasma.		0.8 per cent NaCl.	Calcium added for recalcification.			Time required for complete clotting.	Coagulation observed.
No.	Oxalate concentration.		CaCl ₂ solution.		Oxalate equivalent.		
	<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	<i>cc.</i>		<i>min.</i>	
1	0.25	5	2.5	1	15		Not complete Good. “
	0.25	10	2.5	1	15	9	
	0.25	20	2.5	1	15	5	
	0.25	30	2.5	1	15	6	
	0.25	40	2.5	1	15	7	
2	0.25	10	0.17	2	2	8.5	Good.
	0.25	20	0.17	2	2	8.5	
	0.25	30	0.17	2	2	9	
	0.25	10	0.17	4	4	7	
	0.25	20	0.17	4	4	9.5	
	0.25	30	0.17	4	4	9	Good.
	0.25	10	2.5	1	15	7	
	0.25	20	2.5	1	15	8.5	Good.
	0.25	30	2.5	1	15	9	
1	1.0	10	0.48	5	15	7	
	1.0	20	0.48	5	15	7	
	1.0	30	0.48	5	15	7	
	1.0	40	0.48	5	15	21	

20 and 30 volumes, but with 40 volumes the clot appeared less satisfactory, and the time required for its formation was greater than with 20 or 30 volumes. The use of either 20 or 30 volumes of 0.8 per cent salt solution was, therefore, adopted.

Permissible Range of Calcium Chloride Concentration.—In order to determine the range of calcium concentration over which

satisfactory fibrin clot formation can occur, varying amounts of calcium chloride were added to a series of tubes each containing 1 cc. of plasma and 20 cc. of salt solution.

It is evident from Table IV that fibrin formation is complete if calcium is added in from two to twenty equivalents of the oxalate present. Allowing for a maximum oxalate concentration of 1 per cent, each cc. of plasma would require for an equivalent amount

TABLE IV.

Influence of Concentration of Calcium Chloride on Fibrin Clot Formation.

1 cc. plasma + 20 cc. 0.8 per cent NaCl recalcified in the presence of varying amounts of oxalate.

Oxalate concentration.	Calcium added for recalcification.			Time required for complete clotting.
	CaCl ₂ solution.		Oxalate equivalent.	
per cent	per cent	cc.		min.
1	0.5	0.5	0.37	∞
1	0.5	1	0.75	∞
1	0.5	2	1.5	9.0
1	0.5	4	3.0	5.0
1	2.5	1	3.7	5.5
1	2.5	2	7.5	6.5
1	2.5	4	15.0	11.0
0.25	2.5	0.25	3.7	7.0
0.25	2.5	0.5	7.5	7.5
0.25	2.5	1.0	15.0	7.5
0.25	2.5	1.5	22.5	9.0
0.25	2.5	2.0	30.0	>15
0.25	0.17	0.5	0.5	>30
0.25	0.17	1	1	>30
0.25	0.17	2	2	9
0.25	0.17	4	4	9

of calcium 0.2 cc. of 2.5 per cent CaCl₂. 1 cc. of 2.5 per cent CaCl₂ solution would then contain five equivalents. If the oxalate concentration were 0.25 per cent, which is just sufficient to prevent coagulation, the use of an equal volume of 2.5 per cent CaCl₂ would mean that twenty equivalents of calcium had been used. This amount of CaCl₂ does not interfere with the clot formation, and provides for all probable concentrations of oxalate.

Collection, Washing, and Nitrogen Determination of the Fibrin.—It has been our experience that the determination of the nitrogen content of a protein is a more accurate measure of its mass than the method of drying to constant weight. The errors due to adherent substances or to partial decomposition during drying are thus eliminated. After testing varying conditions for the Kjeldahl digestion the following procedure was adopted. The filter paper with the washed fibrin clot was transferred to a 500 cc. Kjeldahl flask. 20 cc. of concentrated sulfuric acid, 12 gm. of potassium sulfate, and a small crystal of copper sulfate were added, and the mixture was digested for 3 hours after the clearing of the solution.

TABLE V.

Quantitative Test of Fibrin Method.

5 cc. plasma, 0.25 oxalate; 0.8 per cent NaCl solution, CaCl_2 , and washing as indicated. Nitrogen determined by Kjeldahl method.

H_2O	0.8 per cent NaCl added.	Calcium added for recalcification.			Washed.	Fibrin N. per 100 cc.
		CaCl_2 solution.		Oxalate equivalent.		
cc.	cc.	per cent	cc.			gm.
	100	2.5	5	15	0.8 per cent NaCl five times.	0.046
	150	2.5	5	15	0.8 " " " " "	0.044
	150	2.5	10	32	0.8 " " " " "	0.044
	150	1.0	5	6	0.8 " " " " "	0.042
	150	1.0	10	12	0.8 " " " " "	0.044
	50	1.0	10	12	0.8 " " " " "	0.044
	150	1.0	10	12	H_2O till chloride-free.	0.042
	50	1.0	10	12	" " " "	0.048
100		1.0	10	12	" " " "	0.048

The fibrin clot was collected by filtration. In order to be sure that all traces of soluble proteins were removed from the clot, experiments were done to determine whether or not washing with NaCl only or with water was more satisfactory. Each washing was allowed to remain in contact with the clot for several minutes to allow time for diffusion. This was done by closing the outlet at the bottom of the funnel stem for the desired time.

It is evident from Tables V and VI that five washings with salt solution or washing with water until the filtrate is salt-free yields consistent results.

TABLE VI.

Test of Different Methods of Washing Fibrin Clot.

5 cc. plasma diluted to 150 cc. with 0.8 per cent NaCl, 5 cc. 2.5 per cent CaCl₂ solution added. Fibrin washed on filter paper as indicated. Nitrogen determined by Kjeldahl method.

0.8 per cent NaCl.	H ₂ O	Each washing allowed to stand.	Fibrin N per 100 cc.
		min.	gm.
Five times.		10	0.077
" "		10	0.075
" "		10	0.073
" "		15	0.075
" "		30	0.079
	Five times.	30	0.075
	" "	30	0.075
	Three "	30	0.083
	Four "	30	0.075
	" "	60	0.071

TABLE VII.

Influence of Excess of Decalcifying Salts on the Concentration of Total Nitrogen and Fibrin in Plasma.

Blood was drawn mixed with sufficient oxalate to prevent clotting. Salt was then added to portions of blood to concentration indicated. Blood centrifuged and plasma analyzed in usual way.

Sample No.	Concentration of oxalate.	Total N per 100 cc.	Fibrin N per 100 cc.
	per cent	gm.	gm.
1	0.1	1.16	0.024
	1.1	1.018	0.029
	2.0	0.958	0.025
2	0.5	1.0	0.049
	1.0	0.929	0.051
	2.0	0.874	0.051
3	0.5	0.898	0.051
	1.0	0.862	0.047
	2.0	0.746	
To plasma from 0.5 per cent oxalated blood (Sample 3) oxalate was added to make 2 and 5 per cent.	2.0	0.887	
	5.0	0.887	

Influence of the Oxalate Concentration of the Plasma.—It was found (Table VII) that the amount of oxalate present in the plasma had no effect on the fibrin, but that variation in the concentration of oxalate in the whole blood before centrifugation has a considerable effect on the total nitrogen content of the plasma, presumably because of effects on the water distribution between cells and plasma. Consequently in order to obtain accurately comparable results on different plasmas a constant concentration of oxalate must be used. We add 0.5 gm. of potassium oxalate per 100 cc. of whole blood.

SUMMARY.

Methods are described for separation of the fibrin, globulin, and albumin of blood plasma in such a manner that they may be determined by the Kjeldahl method.

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STUDIES OF VARIATIONS IN THE CHEMICAL COMPOSITION OF HUMAN BLOOD.

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INTRODUCTION.

Studies on the chemical composition of human blood have been largely confined to the determination of the nature and the amounts of the constituents. The more exact methods of Folin (1, 2) and his collaborators have placed in the hands of the investigator a means of studying the changes taking place in the concentration of the various blood components in health and disease. The early results have been amplified and extended so that today the literature contains many reports of the normal limits within which these substances fluctuate. Probably the most complete survey of the chemical and physical composition of human blood is that published by Gettler and Baker (3). Studies which have been made of the relations between the determinable nitrogenous constituents have been limited in their applicability by the omission of one or more of the soluble nitrogenous components from direct estimation. The extensive studies of Bang (4) and Feigl (5) are more recent examples of this failure to consider the individual components.

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In view of this lack of tangible data a study is here made of the relative variability and reciprocal relations of the soluble nitrogenous compounds more commonly associated with protein metabolism together with the total nitrogen and the sugar of the blood. The data naturally divide under four headings. There is the study of the relative variability of the blood components for the individual; the discussion of the relative variability of these constituents in a group series of bloods; the influence of the time of withdrawal of the blood on its composition; and lastly, the study of the changes in the amounts, distribution, and interrelations of the nitrogen of the various constituents which accompany the changes in the level of the total non-protein nitrogen itself.

Methods.

The individuals from whom the bloods were taken for these studies were patients and nurses at this hospital. No subject was accepted for study who presented evidence of metabolic disorder. The use of patients suffering from one sort or another of mental disorder for a study of this nature seems to be allowable on the basis of the early findings of Folin (6) that the urinary nitrogenous constituents of such patients are within normal limits. Tracy and Clark (7), however, express the opinion that certain individuals of this type may present some alterations in creatinine excretion due to their relative muscular inactivity.

No quantitative dietary regulation was attempted since the general uniformity of the hospital diet from a qualitative point of view would tend towards the establishment of a qualitative uniformity in its manner of utilization, the degree of which would be dependent ultimately upon the amount of and the individual reaction to the ingested foodstuffs. Any regular variation tendencies that might be found would be all the more indicative of an underlying constancy of metabolic relation. The limits to which data from such material can be used are well recognized and the interpretations that are read into the results are consequently subject to similar conditions and are to be taken with that understanding.

Since the investigations of Raiziss, Dubin, and Ringer (8) indirectly, and of Addis and Watanabe (9), Gettler and Baker (3), and others directly support the contention that a rigid level of content of blood constituents is obtainable only with difficulty and after a considerable period of regulated food intake, the samples of blood to be analyzed were taken at the uniform hour of 11 a.m., $3\frac{1}{2}$ hours after the first meal, unless otherwise noted. The blood from any single individual was not taken more often than once in 7 days.

The blood was drawn into a Record syringe containing a small amount of potassium oxalate (1). Care was taken to avoid the sucking in of air, and after filling the syringe the point of the needle was plunged under paraffin oil (10), and 2 cc. of blood were expressed. This part of the sample was used for the determination of the alkaline reserve. The remainder of the blood, about 25 to 30 cc. in amount, was transferred to a small bottle containing oxalate crystals and brought immediately to the laboratory. From this sample 1 cc. was removed with an Ostwald pipette and diluted to 50 cc. with distilled water in a graduated flask. 1 cc. portions of the diluted blood were used for the estimation of the total nitrogen according to the method of Folin and Farmer (11). The residual sample was freed from protein by the procedure of Folin and Wu (12), and the non-protein nitrogen, urea nitrogen, creatinine, creatine, uric acid, and sugar were determined by following the methods outlined in their publication. The standard for the creatinine and creatine determinations was pure creatinine prepared by the method of Benedict (13). The picric acid was purified according to Folin and Doisy (14), solutions of this substance being made up fresh each day. In the aerations the turpentine and resin mixture of Kendall (15) was used as a foam breaker with good results. The only difficulty that presents itself with this reagent was that there remains a considerable residuum of resinous matter in the test-tubes which must be removed after each total nitrogen determination by boiling with $\text{CuSO}_4\text{-K}_2\text{SO}_4\text{-H}_2\text{SO}_4$ mixture. The amino-acid nitrogen was determined in aliquots of the filtrate after evaporation to a small volume with a few drops of carbonate as indicated by Whipple and Van Slyke (16), the analysis being made with the micro apparatus and technique of the latter investigator (17).

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Preliminary tests showed that the variations in the results obtained in these filtrates from results obtained in the filtrates from other reliable methods of precipitation were negligible. Repeated tests failed to show any marked interfering factor arising from the action of the nitrous acid on the urea during the analysis as carried out and hence no correction was made for this substance. The residual or rest nitrogen was obtained as the difference between the sum of the nitrogen found as urea, creatinine, creatine, uric acid, and amino-acids and that of the total non-protein nitrogen. Separate ammonia determinations were not made. The extreme lability of this substance (18), the observations of Gad-Andersen indicating that the increase in blood ammonia on standing may be due to a concurrent urea decomposition (19), and the confirmation of Folin's (20) statement that the amounts of ammonia in these filtrates are so small as to be negligible, made any attempts at its determination here superfluous.

Individual Variability.

Table I presents the figures for the amounts and the average deviations of each constituent determined in the bloods of nine individuals. The specimens from each individual were taken at weekly intervals $3\frac{1}{2}$ hours after the morning meal, with the exception that Sets 7', 8', and 9' were taken before breakfast. These latter sets are from the same subjects as are the figures in Sets 7, 8, and 9. The results are representative of amounts of nitrogen per 100 cc. of blood.

It will be noted that while the composition of the blood in any given individual varies from week to week the level of the concentrations of the various substances is individual in character.

In Table II are given the sums of the average deviations for each individual. The marked differences observed are taken as meaning differences in metabolic stability.

In order to study the relative variability of the constituents the average deviations were in the case of each blood arranged in the order of increasing value. Since nine constituents are reported there are nine possible places on the scale of ascending variability into which any one of the constituents might fall. Table III gives for each constituent its relative place in the scale of increasing variability in terms of the per cent incidence.

TABLE I.
Individual Variations in Blood Composition.

Set No.	Total N.	Non-protein N.	Urea N.	Creatinine N.	Creatinine N.	Uric acid N.	Amino-acid N.	Rest N.	Sugar.
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	2.6	38.1	21.4	0.48	1.16	0.93	4.1	10.0	88
	3.1	30.6	12.5	0.48	1.35	1.10	6.7	8.5	109
	2.32	30.6	10.6	0.52	1.31	0.77	5.6	11.8	106
	3.06	36.3	17.5	0.44	1.22	1.03	4.8	11.3	105
	3.21	37.8	18.2	0.48	1.15	0.83	4.7	12.4	100
	2.65	31.5	17.6	0.44	1.15	0.70	5.0	6.4	100
Average...	2.83	34.1	16.3	0.47	1.22	0.89	5.1	10.1	101
" deviation	10.6	9.6	19.4	5.0	5.8	14.3	12.8	17.5	5.3
2	2.75	39.0	20.1	0.48	1.22	0.90	4.3	12.0	90
	2.22	39.9	15.1	0.52	1.47	0.70	5.3	16.8	116
	2.91	33.6	17.7	0.44	0.96	0.73	5.7	8.1	92
	2.56	34.8	15.0	0.48	1.52	0.70	4.6	12.5	101
	2.71	33.0	15.4	0.48	1.19	0.70	5.4	9.8	96
	2.80	39.0	21.1	0.48	1.22	0.96	4.5	10.7	97
Average...	2.66	36.5	17.4	0.48	1.26	0.78	4.9	11.6	99
" deviation	6.7	7.5	13.5	2.8	12.2	12.7	10.2	18.3	6.7
3	2.67	43.8	25.1	0.41	1.35	0.77	4.9	11.3	135
	3.09	32.4	15.5	0.48	1.41	0.53	4.8	10.1	120
	2.94	42.9	24.8	0.44	1.22	0.73	6.2	9.5	113
	3.57	41.4	19.5	0.48	1.44	0.70	6.3	13.0	134
Average...	3.09	40.1	21.1	0.45	1.35	0.68	5.5	11.0	125
" deviation	8.5	9.5	18.0	6.1	5.1	11.3	12.7	10.6	7.1
4	3.52	43.3	20.1	0.44	1.25	0.70	4.5	16.2	90
	2.61	38.7	21.2	0.52	1.47	0.73	3.4	11.4	120
	2.61	34.8	15.5	0.41	1.99	0.85	3.6	13.5	87
	2.65	38.4	18.5	0.44	1.78	0.87	3.9	12.9	115
	2.71	37.5	15.9	0.48	1.41	0.83	4.8	14.1	97
	2.80	36.6	22.0	0.41	1.44	0.80	5.4	6.6	100
Average...	2.82	38.2	18.9	0.45	1.39	0.80	4.3	12.4	101
" deviation	8.4	4.8	11.8	7.4	13.0	6.6	14.7	18.7	10.4

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TABLE I—Continued.

Set No.	Total N.	Non-protein N.	Urea N.	Creatinine N.	Creatinine N.	Uric acid N.	Amino-acid N.	Rest N.	Sugar.
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
5	3.24	46.7	13.1	0.41	1.70	0.93	4.6	26.0	105
	2.64	35.4	18.1	0.44	1.35	0.90	5.0	9.3	112
	3.00	34.5	17.7	0.48	1.31	1.07	5.1	8.8	104
	3.00	36.6	13.9	0.44	1.25	1.27	5.7	14.0	126
Average...	2.97	38.3	15.7	0.44	1.40	1.04	5.1	14.5	112
" deviation	5.7	10.9	14.0	3.9	10.5	12.3	5.9	39.5	6.4
6	3.26	40.0	22.6	0.43	1.71	0.57	5.9	8.8	125
	3.30	34.5	17.7	0.40	1.45	0.61	5.6	8.8	122
	2.73	37.3	19.2	0.44	1.44	0.56	6.0	9.6	166
	2.73	33.7	16.7	0.48	1.48	0.73	6.6	7.8	145
Average...	3.00	36.4	19.0	0.44	1.52	0.62	6.0	8.8	139
" deviation	9.2	6.3	9.9	5.1	6.2	9.8	4.8	5.0	11.5
7	3.03	53.6	39.7	0.57	1.42	1.98	6.7	9.4	108
	3.03	55.6	39.0	0.56	1.50	2.14	6.8	10.2	99
	3.00	52.6	37.5	0.62	1.40	2.14	6.4	8.6	92
	3.09	54.5	31.9	0.56	1.27	2.45	6.1	22.4	99
Average...	3.04	54.6	37.0	0.58	1.40	2.18	6.4	12.6	99
" deviation	0.90	1.8	6.9	3.8	4.5	6.3	3.9	38.8	4.0
8	3.33	33.7	16.6	0.46	1.20	0.79	6.1	8.6	89
	3.12	33.1	15.9	0.48	1.42	0.79	3.6	11.0	133
	3.30	34.6	13.1	0.43	1.19	0.70	5.3	13.9	96
	3.42	38.7	16.9	0.50	1.25	0.96	5.0	14.1	115
Average...	3.29	35.0	15.6	0.47	1.26	0.81	5.0	11.9	108
" deviation	2.6	5.2	8.0	4.8	6.0	9.3	13.9	17.7	14.8
9	3.66	35.9	23.6	0.54	1.49	0.69	6.9	3.7	114
	3.15	45.5	23.8	0.54	1.49	0.82	6.3	12.5	106
	3.00	40.0	22.1	0.53	1.30	0.99	5.3	9.8	103
	2.88	37.3	21.3	0.53	1.38	0.80	5.7	7.6	111
Average...	3.17	39.7	22.7	0.53	1.41	0.82	5.8	8.4	108
" deviation	7.7	7.6	4.3	1.0	5.2	9.8	5.0	32.8	3.7

TABLE I—*Concluded.*

Set No.	Total N.	Non-protein N.	Urea N.	Creatinine N.	Crea-tine N.	Uric acid N.	Amino-acid N.	Rest N.	Sugar.
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
7'	2.88	56.6	33.0	0.53	1.48	2.03	5.9	13.7	119
	3.30	52.2	31.9	0.52	1.51	2.00	5.6	10.6	116
	2.97	50.9	30.0	0.62	1.35	2.28	6.5	10.1	87
	3.00	54.5	30.6	0.53	1.31	1.74	5.6	14.7	118
Average...	3.04	53.5	31.4	0.55	1.41	2.01	5.8	12.3	110
" deviation	4.3	3.7	3.3	6.8	5.8	7.2	2.4	15.5	10.3
8'	3.30	32.8	14.2	0.46	1.28	0.78	5.0	11.0	100
	3.45	31.4	16.7	0.46	1.40	0.73	5.1	7.1	105
	3.24	37.1	14.2	0.48	1.21	0.66	5.6	14.9	104
	3.16	36.2	14.8	0.48	1.31	0.67	5.4	13.6	108
Average...	3.29	34.4	15.0	0.47	1.30	0.71	5.3	11.7	103
" deviation	2.6	6.6	5.9	2.0	2.9	6.3	4.2	14.6	2.5
9'	3.33	36.2	19.8	0.49	1.20	0.76	4.8	9.1	138
	3.00	32.9	18.9	0.45	1.46	0.58	4.9	7.5	126
	3.33	36.7	22.6	0.48	1.46	0.51	6.3	5.1	97
	3.00	30.6	19.1	0.48	1.31	0.57	5.7	3.4	96
Average...	3.16	34.1	20.1	0.47	1.36	0.60	5.4	6.2	114
" deviation	5.2	6.8	6.2	3.1	7.5	12.4	10.6	32.2	15.5

TABLE II.

Relative Metabolic Stability of Different Individuals as Calculated from Average Deviations Observed in Table I.

Set No.....	1	2	3	4	5	6	7	8	9	7'	8'	9'
Relative stability.....	100	91	89	96	109	68	71	82	77	59	48	101

Table III reveals a quite consistent tendency to increasing variability of the constituents in the order given, the scatterings that occur being due in large part to individual differences in nature of metabolic stability.

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Group Variability.

We turn now from the consideration of the variations in the constituents of the blood of an individual to a study of the variations within a group of bloods. Although the literature contains many reports and controversies concerning the limits that may be considered as normal for the various substances determined, it is not the purpose of this study to enter into any discussion of the relative merits of the results of the various investigators or to set new limits or to confirm old ones. That no hard and fast lines can be drawn for the limits within which the amounts of any individual component may be supposed to fall to

TABLE III.
Order of Variability of Blood Constituents in the Individual.

	1	2	3	4	5	6	7	8	9
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Creatinine N.	50	25	17			8			
Total N.	17	25	8	25	8	8	8		
Non-protein N.	8	8	17	17	17	25		8	
Creatine N.	8		8	25	33	17	8		
Sugar.		25	8	8	17			25	17
Amino-acid N.	17		8	17	8	17	17	17	
Uric acid N.		8			8	8	58	17	
Urea N.		8	17		8	17	8	25	17
Rest N.			17			8		8	67

be considered normal in amount is amply evidenced by the studies of Addis and Watanabe (21), McLean and Selling (22), and others. The conservative opinion seems to be that except in extreme cases it is the blood picture as a whole that is indicative of a pathological condition, rather than the amount of any particular constituent which for the moment may be exhibiting an abnormality that may be more apparent than real.

In order to save space and repetition Table IV is limited to the figures representing the range within which the various substances fluctuate, the averages of the amounts found, the average deviation for each constituent, and the relative variability. The absolute values are given in terms of nitrogen per 100 cc. of blood and are arranged in the order of their increasing variability.

In general it can be said that a comparison of the range of variation observed in these results with the reported findings of other workers shows a general agreement in values. It is to be remembered that the figures represent the extreme limits found and that where they appear to differ materially from the so called normal limits they nevertheless may properly be considered as within what might be expected in the sense of the theory of probability.

An inspection of the average deviations makes evident that they are not simply related to the absolute amounts of the respective substances but rather express differences in the susceptibility of the constituents to variation. A comparison of the

TABLE IV.

Range of Variation, Average Amounts, Average Deviations, and Relative Variability of the Blood Constituents Determined.

	Range.	Average.	Average deviation.	Relative variability.
	mg.	mg.	per cent	
Creatinine N.....	0.37-0.60	0.47	7.3	100
Non-protein N.....	27.3-45.5	35.6	9.1	125
Total N.....	2560-4290	307.0	9.4	129
Creatine N.....	0.62-1.78	1.31	11.5	158
Sugar.....	85-166	112.0	13.7	188
Uric acid N.....	0.50-1.16	0.78	14.9	204
Amino-acid N.....	3.1-7.2	4.9	15.3	210
Urea N.....	9.7-25.1	17.1	16.6	227
Rest N.....	3.7-18.3	11.1	20.1	275

order of variation here obtained with that shown in Table III demonstrates that no significant changes have occurred, the interchange of position of the total nitrogen and the non-protein nitrogen, as well as that occurring in the amino-acid and uric acid nitrogen, being due to quantitatively negligible differences.

Such being the case we have an interesting sequence in variability of the constituents under investigation. The causes of these differences in variability cannot be attributed solely to differences in amounts or nature of absorbed material since the order of variability tends to be the same in different individuals presumably ingesting quantitatively different diets. Nor can these differences be wholly ascribed to individual differences in

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metabolic stability. It would appear as if the order of variability here manifested is rather the expression of an underlying uniformity of metabolic mechanism specific for the species.

The significance of the position of the various constituents in Table IV is not wholly obvious. They can be, however, divided roughly into three groups, one of relatively low variability containing the creatinine, the total non-protein nitrogen, and the total nitrogen; one of intermediate variability, into which fall the creatine and sugar; and one of relatively high variability containing the uric acid, amino-acid, and urea nitrogen.

The fact that the creatinine appears to be the least susceptible to variation would lend additional support to the conception of Folin and Denis (23) that this substance is the result of a uniform endogenous process and that it represents a certain particular form of protein metabolism.

The relatively low variability of the total non-protein nitrogen is in part accounted for by compensatory variations in deviation occurring between the urea nitrogen, the amino-acid, and rest nitrogen. The variability of the total nitrogen is probably due as much to differences in water intake as to individual differences (24).

The differences in the stability of the metabolic factors leading to the production of creatinine and creatine are well emphasized in the 60 per cent greater variability of the latter.

The factors concerned in the regulation of the amounts of sugar in the blood have been quite fully discussed in a recent publication by Grote (25). It is evident from Table IV that the sugar variability lies near the middle of the scale.

The relatively high variability of the uric acid is probably due to variations in the activity of the endogenous processes giving rise to this blood constituent. According to Mareš (26) these processes are largely confined to the nuclei of the digestive glands, but recent work of Lewis, Dunn, and Doisy (27) has shown that there is a general nuclear activity induced by a stimulative effect of the amino-acids. That the source of the variability is endogenous is strengthened by the findings of Denis (28) that the concentration of uric acid in the blood does not rise in normal individuals during the ingestion of purine-containing foods.

Both Folin and Denis (29) and Van Slyke and Meyer (30) have shown the existence of an interrelation between the metabolic factors concerned with the concentration of amino-acids and urea in the blood, and in view of our present ideas concerning their metabolism a relatively high variability in their concentration is not unexpected. The rest nitrogen will be considered later.

Influence of Time of Withdrawal of Blood on Its Composition.

Although Gettler and Baker (3) state that the concentrations of the various components of the blood fall within normal limits

TABLE V.

Differences in Concentration of Total Nitrogen, Non-Protein Nitrogenous Constituents, and Sugar in Bloods Taken before and after Breakfast.

	Total N.	Non-protein N.	Urea N.	Creatinine N.	Creatinine N.	Uric acid N.	Amino-acid N.	Rest N.	Sugar.
Sets 7-7'.									
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Before	3.04	53.5	31.4	0.55	1.41	2.01	5.8	12.3	110
After	3.04	54.6	37.0	0.58	1.40	2.18	6.4	12.6	99
Sets 8-8'.									
Before	3.29	34.4	15.0	0.47	1.30	0.71	5.3	11.7	103
After	3.29	35.0	15.6	0.47	1.26	0.81	5.0	11.9	108
Sets 9-9'.									
Before	3.17	34.1	20.1	0.47	1.36	0.60	5.4	6.2	114
After	3.17	39.7	22.7	0.53	1.41	0.82	5.8	8.4	108

whether the blood is taken before or 3 hours after breakfast, and Addis and Watanabe (21) confirm this for urea, it is advisable to make a detailed comparison of bloods taken from the same individuals before and $3\frac{1}{2}$ hours after breakfast for obvious practical reasons. In Table I Sets 7, 8, and 9 are from bloods taken at weekly intervals $3\frac{1}{2}$ hours after the morning meal. Sets 7', 8', and 9' are bloods from the same individuals in the order given, taken at weekly intervals before breakfast. To facilitate comparison Table V presents the data in a condensed form. The absolute amounts are the averages for the four weekly determinations on each individual.

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It is seen that there is no practical difference in the concentration of the various constituents determined in blood taken 14 and 3½ hours after eating. From the metabolic point of view the blood taken before breakfast shows evidence of a slightly lower metabolism at this period. The non-protein nitrogen, the urea nitrogen, and the uric acid nitrogen are consistently lower, but to a very small extent. It is seen that, as others have shown in similar comparisons, the sugar may be somewhat higher after the longer fast (31). The changes in the average deviations are generally indeterminate.

Nitrogen Distribution in the Blood.

Our present conception of protein metabolism is largely derived from the studies of Folin (32) on the laws governing the chemical composition of the urine. These studies are based on the differences in the nitrogen and sulfur distribution in urines of high and low total nitrogen content. The sharp differences observed in the nitrogen distribution in such urines, however, cannot be expected in the blood. There is, however, a definite tendency for a change in nitrogen distribution to take place with changes in the level of the total non-protein nitrogen. In Table VI are given the analyses previously summarized in Table IV, as nitrogen per 100 cc. of blood, and the per cent of nitrogen of each constituent in terms of the total non-protein nitrogen. The values for the individual samples of blood have been arranged in the order of descending values of the non-protein nitrogen.

With a decrease in the value of the total non-protein nitrogen there is a general tendency for a simultaneous decrease in the absolute amounts of the urea, creatine, uric acid, and amino-acid nitrogen, of which the urea reduction is the most uniform. There are differences in the degree to which these constituents decrease with the non-protein nitrogen, the urea showing relatively the greater diminution. The rest nitrogen seems to be less affected by a change in the non-protein nitrogen level.

The lack of definite change in the concentration of creatinine nitrogen is significant in that the concentration is thus shown to be independent of that of the total non-protein nitrogen.

TABLE VI.

The Amounts and Percentages in Terms of Non-Protein Nitrogen of the Non-Protein Nitrogenous Constituents of 100 Cc. of Human Blood.

No.	Non-protein N.		Urea N.		Creatinine N.		Creatine N.		Uric acid N.		Amino-acid N.		Rest N.	
	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
1	45.5	23.8	52.4	0.54	1.2	1.49	3.3	0.82	1.8	6.3	13.8	12.5	27.6	
2	43.8	25.1	57.3	0.41	0.9	1.35	3.2	0.77	1.8	4.9	11.2	11.3	25.8	
3	43.2	20.1	46.5	0.44	1.0	1.25	2.9	0.70	1.6	4.5	10.4	16.2	37.5	
4	42.9	24.8	57.8	0.44	1.0	1.22	2.8	0.73	1.7	6.2	14.5	9.5	22.1	
5	42.3	20.0	47.3	0.52	1.2	1.25	3.2	0.77	1.8	5.4	12.8	14.2	33.6	
6	41.4	19.5	47.1	0.48	1.2	1.44	3.5	0.70	1.7	6.3	15.2	13.0	31.4	
7	40.5	15.6	38.5	0.44	1.1	1.57	3.9	0.77	1.9	3.9	9.6	18.3	45.2	
8	40.0	22.1	55.2	0.53	1.3	1.30	3.3	0.99	2.5	5.4	13.3	9.8	24.5	
9	40.0	22.6	56.4	0.43	1.1	1.71	4.3	0.57	1.4	5.9	14.8	8.8	22.0	
10	39.0	20.1	51.5	0.48	1.2	1.22	3.1	0.90	2.3	4.3	11.0	12.0	30.7	
11	39.0	21.1	54.1	0.48	1.2	1.22	3.1	0.96	2.5	4.5	11.6	10.7	27.4	
12	38.7	21.1	54.8	0.52	1.3	1.47	3.8	0.73	1.7	3.4	8.8	11.4	29.5	
13	38.7	16.9	43.5	0.50	1.3	1.25	3.2	0.96	2.5	5.0	12.9	14.1	36.5	
14	38.7	19.8	51.1	0.60	1.6	1.25	3.2	0.83	2.1	4.5	11.6	11.7	30.2	
15	38.4	17.6	45.8	0.48	1.3	1.41	4.1	1.16	3.0	4.7	12.1	13.1	34.1	
16	38.4	18.5	48.2	0.44	1.1	1.78	4.6	0.87	2.3	3.9	10.2	12.9	33.6	
17	38.1	21.4	56.1	0.48	1.3	1.16	3.1	0.93	2.4	4.1	10.8	10.0	26.3	
18	37.8	18.2	48.3	0.48	1.3	1.15	3.0	0.83	2.2	4.7	12.4	12.4	32.8	
19	37.5	15.9	42.4	0.48	1.3	1.41	3.8	0.83	2.2	4.8	12.8	14.1	37.6	
20	37.5	15.5	41.3	0.52	1.4	1.22	3.3	0.73	1.9	6.1	16.3	13.4	35.7	
21	37.3	21.3	57.1	0.53	1.4	1.38	3.7	0.82	2.2	5.7	15.3	7.6	20.4	
22	37.3	19.2	51.6	0.44	1.2	1.44	3.9	0.56	1.5	6.0	16.0	9.6	25.8	
23	36.6	22.0	60.1	0.41	1.1	1.44	3.9	0.80	2.2	5.4	14.7	6.6	18.0	
24	36.3	17.5	48.2	0.44	1.2	1.22	3.4	1.03	2.8	4.8	13.2	11.3	31.1	
25	36.0	13.3	37.0	0.44	1.2	1.60	4.4	1.00	2.8	7.2	20.0	12.5	34.7	
26	35.9	15.3	42.6	0.48	1.3	1.06	3.0	0.67	1.9	4.1	11.4	14.3	38.8	
27	35.9	23.6	65.7	0.54	1.5	1.48	4.1	0.69	1.9	5.9	16.5	3.7	10.4	
28	35.7	16.4	45.9	0.44	1.2	1.31	3.7	0.70	2.0	4.8	13.4	12.0	33.6	
29	35.4	18.4	52.0	0.44	1.2	1.35	3.8	0.90	2.5	5.0	14.1	9.3	26.3	
30	34.8	15.0	43.1	0.48	1.4	1.52	4.4	0.70	2.0	4.6	13.2	12.5	35.9	
31	34.8	15.5	44.3	0.41	1.2	0.99	2.8	0.83	2.4	3.6	10.3	13.6	38.8	
32	34.6	13.1	37.9	0.43	1.2	1.19	3.4	0.70	2.0	5.3	15.3	13.9	40.2	
33	34.5	17.7	51.3	0.48	1.4	1.31	3.8	1.07	3.1	5.1	14.8	8.8	25.6	
34	34.5	17.7	51.2	0.40	1.2	1.45	4.2	0.61	1.8	5.6	16.1	8.8	25.6	
35	34.2	11.8	34.5	0.44	1.3	0.96	2.5	0.70	2.1	3.7	10.8	16.6	48.6	
36	33.9	15.1	44.5	0.48	1.4	0.93	2.7	0.80	2.3	5.3	15.6	11.2	33.0	
37	33.7	16.6	49.2	0.46	1.4	1.20	3.6	0.79	2.3	6.1	18.1	8.6	25.5	
38	33.7	16.7	49.5	0.48	1.4	1.48	4.4	0.73	2.2	6.6	19.5	7.8	23.1	

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TABLE VI—*Concluded.*

No.	Non-protein N.			Urea N.		Creatinine N.		Creatine N.		Uric acid N.		Amino-acid N.		Rest N.	
	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
39	33.6	17.7	52.7	0.44	1.3	0.96	2.9	0.73	2.3	5.7	17.0	8.1	24.3		
40	33.6	11.1	33.0	0.41	1.2	1.38	4.1	0.63	1.9	4.0	11.9	16.1	48.1		
41	33.6	13.3	39.6	0.52	1.5	1.35	4.1	1.16	3.5	5.6	16.7	11.6	34.5		
42	33.1	15.9	47.9	0.48	1.4	1.42	4.3	0.79	2.4	3.6	10.9	11.0	33.1		
43	33.0	12.2	37.0	0.52	1.6	1.38	4.2	0.67	2.0	4.0	12.1	14.2	43.0		
44	33.0	15.4	46.7	0.48	1.5	1.19	3.6	0.70	2.1	5.4	16.4	9.8	29.8		
45	33.0	12.3	37.3	0.52	1.6	1.35	4.2	0.77	2.3	4.0	12.1	14.1	42.7		
46	32.8	18.3	55.8	0.48	1.5	1.35	4.1	0.70	2.1	3.6	11.0	8.4	25.6		
47	32.4	16.6	51.3	0.48	1.5	1.60	4.9	0.67	2.1	5.3	16.4	7.7	23.8		
48	32.4	14.8	46.0	0.41	1.3	1.47	4.5	0.50	1.5	4.3	13.3	10.8	33.3		
49	32.4	15.1	46.6	0.48	1.5	1.41	4.4	0.53	1.6	4.8	14.8	10.1	31.5		
50	32.4	16.3	50.3	0.52	1.6	1.12	3.5	0.70	2.2	3.9	12.0	9.9	30.6		
51	32.1	16.6	51.7	0.44	1.4	1.16	3.6	0.87	2.7	4.7	14.6	8.3	25.9		
52	31.8	12.8	40.3	0.44	1.4	1.38	4.3	0.73	2.5	5.0	15.7	11.4	35.8		
53	31.7	17.0	53.7	0.43	1.4	1.03	3.3	0.60	1.9	3.9	12.4	8.7	27.4		
54	31.5	17.6	55.9	0.44	1.4	1.15	3.6	0.70	2.2	5.0	15.9	6.6	20.9		
55	30.6	12.5	40.9	0.48	1.6	1.35	4.4	1.10	3.6	6.7	21.9	8.5	27.8		
56	30.0	14.4	48.0	0.48	1.6	1.16	3.9	0.83	2.8	4.1	13.7	9.0	30.0		
57	29.7	13.3	44.1	0.41	1.4	1.25	4.2	0.90	3.0	3.1	10.4	10.9	36.7		
58	28.8	11.3	39.2	0.48	1.7	1.28	4.4	0.73	2.5	5.0	17.4	10.0	34.7		
59	28.2	15.8	55.9	0.48	1.7	0.63	2.2	0.65	2.3	3.9	13.9	6.7	23.8		
60	27.3	9.7	35.5	0.37	1.4	1.35	4.9	0.57	2.1	4.3	15.8	11.0	40.3		
Average...	35.6	17.1	47.8	0.47	1.3	1.30	3.7	0.78	2.2	4.9	13.8	11.0	31.1		

If instead of the concentrations we consider the percentages of the total non-protein nitrogen which they represent, it is evident that in the blood as in the urine (33) the distribution of the nitrogen among the urea and the other nitrogenous constituents is dependent upon the absolute amounts of the total non-protein nitrogen present. For not only does the urea nitrogen tend to decrease in absolute amounts but also the percentage of the total non-protein nitrogen as urea nitrogen tends to decrease with the fall in the absolute amounts of the total non-protein nitrogen. This fall in percentage of urea nitrogen indicates that with falling total non-protein nitrogen the blood urea tends to decrease in concentration to a relatively greater extent than do the other constituents.

In order to bring out the existence of any specific relations between one constituent and the others, the absolute amounts of each component were arranged in the order of their descending value without regard to origin and compared with the corresponding values of the other constituents. It was found that when the urea nitrogen was used as the basis for comparison the only significant relation to be observed lay in the fact that there was a tendency for the rest nitrogen to increase in absolute amounts with the decrease in the urea nitrogen.

Similar comparisons using the creatinine, creatine, uric acid, and amino-acid nitrogen respectively as a basis have revealed no significant interrelations. The ratio between the creatinine and creatine nitrogen is quite variable, the creatinine nitrogen ranging from 24 to 76 per cent of the creatine nitrogen as the extreme limits, the common limits being from 27 to 46 per cent. About 13 per cent of the values of the ratio lie between 24 and 28 per cent, 77 per cent between 30 and 45 per cent, and 10 per cent between 46 and 76 per cent.

SUMMARY.

These studies of the chemical composition of human blood indicate:

1. While the total nitrogen, non-protein nitrogenous constituents, and the sugar of the blood vary in the same individual from week to week, there is a tendency for the level of these variations to be characteristically individual.

2. The sum of the average deviations of the constituents for any given individual may be an index of the metabolic stability of that individual.

3. The order of relative variability of the constituents determined is: creatinine, total non-protein nitrogen, total nitrogen, creatine, sugar, uric acid, amino-acid, urea, and rest nitrogen. A rough division can be made into groups of low, intermediate, and high variability.

4. There is no practical difference between the absolute amounts of the constituents found in bloods taken 14 hours after eating, *i.e.* before breakfast, and 3½ hours after this meal. The slightly lower values found before breakfast for the non-protein, urea, and uric acid nitrogen are taken as meaning a lessened metabolism.

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5. The absolute amounts of the urea, creatine, uric acid, amino-acid, and rest nitrogen tend to decrease with decrease in level of the total non-protein nitrogen. The urea decreases to relatively the greatest extent.

6. The absolute amount of creatinine is a constant for the individual and for the species. It is independent of quantitative changes in the level of the non-protein nitrogen; of the changes in the concentrations of any of the other constituents determined; and of the individual variations in metabolic stability.

7. There is no uniform quantitative relation between the amounts of the creatinine and creatine nitrogen in the blood.

8. The distribution of the non-protein nitrogen among urea and the other soluble nitrogenous constituents of the blood is dependent to a great degree upon the absolute amounts of the total non-protein nitrogen present.

9. The nitrogenous constituents of blood that are commonly found in the urine, *e.g.* urea, creatinine, and uric acid, seem to undergo the same type of absolute and relative change with change in level of the total non-protein nitrogen of the blood as they do with the change from the high to low total nitrogen in the urine.

10. The relative as well as the absolute decline in the urea nitrogen of the blood accompanying the decrease in the total non-protein nitrogen is compensated for by a relatively lesser absolute decrease in all the other nitrogenous constituents, which results in an increase of the percentage of their nitrogen in terms of the non-protein nitrogen.

11. The only apparent interrelation existing between any of the individual constituents is that between the urea and the rest nitrogen. There is a tendency for a rise or fall in the blood urea to be accompanied by a change in the opposite direction of the rest nitrogen.

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THE HEAT COAGULATION OF MILK.

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In our article with the above title published in this *Journal* in November, 1919, the temperature of 136°C. was chosen as a suitable one for the detection of milks liable to coagulate in the condensing process. Mr. George Grindrod, now with the Carnation Milk Products Company of Oconomowoc, Wisconsin, was the first to employ high temperatures in a "heat test" as suitable for the detection of such condition and to have used such temperatures with the autoclave. In our paper we failed to give Mr. Grindrod credit for having made use of this means of testing milk for coagulation effects. We now wish to correct this error of omission.

In our paper we mentioned the disadvantage of using the autoclave for heating the milk and reported on our modification of using sealed tubes in a xylene bath at 136°C. In our later correspondence with Mr. Grindrod he claims that he had also used the sealed tube method for a period of 3 years, although he had not published any description of this test or given us any information of a similar import.

A NEW 0.1 N CALOMEL ELECTRODE DESIGN.

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In accurate H ion concentration work the 0.1 N calomel electrode is nearly universally used, mainly because its E. M. F. is not nearly so readily affected by temperature change as the saturated type.

Furthermore, a saturated KCl solution, unless in a tightly sealed vessel, is disagreeable to work with because of the tendency of the salt to creep from the solution.

However, it is apparent that the 0.1 N electrode can be used to advantage only when the normality remains perfectly constant. When the 0.1 N electrode is used with the necessary saturated KCl bridge, this becomes a problem.

Various appliances are in use and practically all depend on a very small opening at the end of the side-arm of the calomel electrode. Diffusion is only hindered at best and the arm has to be washed out with the solution from the electrode vessel. Furthermore, the side-arm must remain in the saturated KCl solution only when necessary, so the arrangement is not permanent.

We are using a design in several H ion concentration apparatus which seems to overcome these difficulties completely. Its use was practically imperative in a problem where several determinations were made each day over a long period of time, inasmuch as the electrode chain could be set up permanently without any change in normality of the 0.1 N KCl or any creeping of salts.

During a period of nearly 2 months of continual use the E. M. F. of the calomel electrode had not changed in the least.

The design can readily be understood from the diagram. The apparatus is clamped to a horizontal bar and the lower tube from stop-cock C connected by a large flexible rubber tube to

the Clark or McClendon electrode mounted on the usual shaking arm. The vertical arm from stop-cock C is connected to a bottle of saturated KCl solution which usually is placed well back of the apparatus so as to be out of the way. The same is true of the bottle of 0.1 N KCl solution which is connected to the other vertical arm.

Contact is made between the 0.1 N KCl and the saturated KCl solution around the closed, ungreased stop-cock B. Stop-cock A, which is greased with pure vaseline, is opened so as to connect the

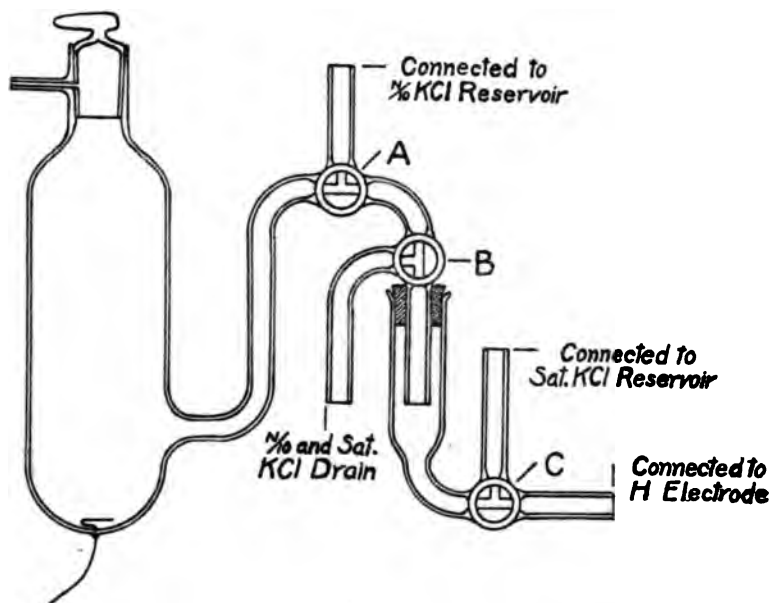


FIG. 1

electrode vessel with the bridge *only* when making a reading. Immediately after making a reading by the proper manipulation of stop-cocks A and B the section from A to B is thoroughly washed out through the side drain arm with fresh 0.1 N KCl from the reservoir.

In a similar manner any 0.1 N KCl can be washed from the saturated KCl below stop-cock B.

This apparatus thus gives a permanently closed system which minimizes contamination of the 0.1 N solution of the calomel electrode.

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